CORRECTED
VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: ADJUVANT FOR TRANSCUTANEOUS IMMUNIZATION

(57) Abstract

A transcutaneous immunization system delivers antigen to immune cells without perforation of the skin, and induces an immune response in an animal or human. The system uses an adjuvant, preferably an ADP-ribosylating exotoxin, to induce an antigen-specific immune response (e.g., humoral and/or cellular effectors) after transcutaneous application of a formulation containing antigen and adjuvant to intact skin of the animal or human. The efficiency of immunization may be enhanced by adding hydrating agents (e.g., liposomes), penetration enhancers, or occlusive dressings to the transcutaneous delivery system. This system may allow activation of Langerhans cells in the skin, migration of the Langerhans cells to lymph nodes, and antigen presentation.

ADJUVANT FOR TRANSCUTANEOUS IMMUNIZATION

BACKGROUND OF THE INVENTION

The invention relates to transcutaneous
immunization, and adjuvants useful therein, to induce

5   an antigen-specific immune response.

Transcutaneous immunization requires both passage
of an antigen through the outer barriers of the skin,
which are normally impervious to such passage, and an
immune response to the antigen. In U.S. Appln. No.

10   08/749,164, use of cholera toxin as an antigen was
shown to elicit a strong antibody response that is
highly reproducible; the antigen could be applied in a
saline solution to the skin, with or without
liposomes. In the present application, we show

15   transcutaneous immunization using adjuvants such as,
for example, bacterial exotoxins, their subunits, and
related toxins.

There is a report of transdermal immunization
with transferosomes by Paul et al. (1995). In this

20   publication, the transferosomes are used as a carrier
for proteins (bovine serum albumin and gap junction
proteins) against which the complement-mediated lysis
of antigen-sensitized liposomes is directed. An
immune response was not induced when solution

25   containing the protein was placed on the skin; only
transferosomes were able to transport antigen across
the skin and achieve immunization. As discussed in
U.S. Appln. No. 08/749,164, transferosomes are not
liposomes.

30   Figure 1 of Paul et al. (1995) showed that only a
formulation of antigen and transferosomes induced an
immune response, assayed by lysis of antigen-
sensitized liposomes. Formulations of antigen in
solution, antigen and mixed micelles, and antigen and

35   liposomes (i.e., smectic mesophases) applied to the

skin did not induce an immune response equivalent to
that induced by subcutaneous injection. Therefore,
there was a positive control (i.e., antigen and
transfersomes) to validate their negative conclusion
5 that a formulation of antigen and liposomes did not
cause transdermal immunization.

Paul et al. (1995) stated on page 3521 that the
skin is an effective protective barrier that is
"impenetrable to substances with a molecular mass at
10 most 750 DA", precluding non-invasive immunization
with large immunogen through intact skin. Therefore,
the reference would teach away from using a molecule
like cholera toxin (which is 85,000 daltons) because
such molecules would not be expected to penetrate the
15 skin and, therefore, would not be expected to achieve
immunization. Thus, skin represents a barrier that
would make penetration by an adjuvant or antigen like
cholera toxin unexpected without the disclosure of the
present invention.

20 Paul and Cevc (1995) stated on page 145, "Large
molecules normally do not get across the intact
mammalian skin. It is thus impossible to immunize
epicutaneously with simple peptide or protein
solutions." They concluded, "The dermally applied
25 liposomal or mixed micellar immunogens are
biologically as inactive as simple protein solutions,
whether or not they are combined with the
immunoadjuvant lipid A."

Wang et al. (1996) placed a solution of ovalbumin
30 (OVA) in water on the skin of shaved mice to induce an
allergic type response as a model for atopic
dermatitis. Mice were anesthetized and covered with
an occlusive patch containing up to 10 mg of OVA,
which was placed on the skin continuously for four
35 days. This procedure was repeated after two weeks.

In Figure 2 of Wang et al. (1996), an ELISA assay
done to determine the IgG2a antibody response showed

3

no IgG2a antibody response to OVA. However, IgE
antibodies that are associated with allergic responses
could be detected. In a further experiment, the mice
were more extensively patched with OVA in solution for

5   four days every two weeks. This was repeated five
times, i.e., the mice wore patches for a total of 20
days. Again, the high dose of OVA did not produce
significant IgG2a antibodies. Significant levels of
IgE antibodies were produced.

10   The authors stated on page 4079 that "we
established an animal model to show that epicutaneous
exposure to protein Ag, in the absence of adjuvant,
can sensitize animals and induce a dominant Th2-like
response with high levels of IgE". Extensive

15 epicutaneous exposure to high doses of protein antigen
could not produce significant IgG antibodies but could
induce IgE antibodies, the hallmark of an allergic
type reaction. Thus, Wang et al. (1996) teaches that
OVA exposure as described is a model for atopic

20 dermatitis and not a mode of immunization. Therefore,
following the teaching of the reference, one would
have expected that transcutaneous immunization with
antigen would induce high levels of IgE antibodies if
it were to pass through the skin and induce an immune

25 response. Instead, we have unexpectedly found that
antigen placed on the skin in a saline solution with
adjuvant induces high levels of IgG and some IgA, but
not IgE.

        In contrast to the cited references, the
30 inventors have found that application to the skin of
antigen and adjuvant provides a transcutaneous
delivery system for antigen that can induce an
antigen-specific immune response of IgG or IgA. The
adjuvant is preferably an ADP-ribosylating exotoxin.

35 Optionally, hydration, penetration enhancers, or
occlusive dressings may be used in the transcutaneous
delivery system.

4

SUMMARY OF THE INVENTION

An object of the invention is to provide a system for transcutaneous immunization that induces an immune response (e.g., humoral and/or cellular effectors) in
5   an animal or human.

The system provides simple application to intact skin of an organism of a formulation comprised of antigen and adjuvant to induce a specific immune response against the antigen.

10   In particular, the adjuvant may activate antigen presenting cells of the immune system (e.g., Langerhans cells in the epidermis, dermal dendritic cells, dendritic cells, macrophages, B lymphocytes) and/or induce the antigen presenting cells to
15   phagocytose the antigen. The antigen presenting cells then present the antigen to T and B cells. In the instance of Langerhans cells, the antigen presenting cells then may migrate from the skin to the lymph nodes and present antigen to lymphocytes (e.g., B
20   and/or T cells), thereby inducing an antigen-specific immune response.

In addition to eliciting immune reactions leading to generation of an antigen-specific B lymphocyte and/or T lymphocyte, including a cytotoxic T
25   lymphocyte (CTL), another object of the invention is to positively and/or negatively regulate components of the immune system by using the transcutaneous immunization system to affect antigen-specific helper T lymphocytes (Th1, Th2 or both).

30   In a first embodiment of the invention, a formulation containing antigen and adjuvant is applied to intact skin of an organism, the antigen is presented to immune cells, and an antigen-specific immune response is induced without perforating the
35   skin. The formulation may include additional antigens such that transcutaneous application of the

5

formulation induces an immune response to multiple
antigens. In such a case, the antigens may or may not
be derived from the same source, but the antigens will
have different chemical structures so as to induce

5   immune responses specific for the different antigens.
Antigen-specific lymphocytes may participate in the
immune response and, in the case of participation by B
lymphocytes, antigen-specific antibodies may be part
of the immune response.

10   In a second embodiment of the invention, the
above method is used to treat an organism. If the
antigen is derived from a pathogen, the treatment
vaccinates the organism against infection by the
pathogen or against its pathogenic effects such as

15   those caused by toxin secretion. A formulation that
includes a tumor antigen may provide a cancer
treatment, a formulation that includes an autoantigen
may provide a treatment for a disease caused by the
organism's own immune system (e.g., autoimmune

20   disease), and a formulation that includes an allergen
may be used in immunotherapy to treat an allergic
disease.
         In a third embodiment of the invention, a patch
for use in the above methods is provided. The patch

25   comprises a dressing, and effective amounts of antigen
and adjuvant. The dressing may be occlusive or non-
occlusive. The patch may include additional antigens
such that application of the patch induces an immune
response to multiple antigens. In such a case, the

30   antigens may or may not be derived from the same
source, but the antigens will have different chemical
structures so as to induce an immune response specific
for the different antigens. For effective treatment,
multiple patches may be applied at frequent intervals

35   or constantly over a period of time.
         Moreover, in a fourth embodiment of the
invention, the formulation is applied to intact skin

6

overlying more than one draining lymph node field
using either single or multiple applications. The
formulation may include additional antigens such that
application to intact skin induces an immune response
5   to multiple antigens. In such a case, the antigens
may or may not be derived from the same source, but
the antigens will have different chemical structures
so as to induce an immune response specific for the
different antigens.

10   The products and methods of the invention may be
used to treat existing disease, to prevent disease, or
to reduce the severity and/or duration of disease.
However, induction of allergy, atopic disease,
dermatitis, or contact hypersensitivity is not
15 preferred.

In addition to antigen and adjuvant, the
formulation may further comprise a hydrating agent
(e.g., liposomes), a penetration enhancer, or both.
For example, the antigen-adjuvant formulation may
20 further comprise an emulsion made with AQUAPHOR
(petrolatum, mineral oil, mineral wax, wool wax,
panthenol, bisabol, and glycerin), emulsions (e.g.,
aqueous creams), oil-in-water emulsions (e.g., oily
creams), anhydrous lipids and oil-in-water emulsions,
25 anhydrous lipids and water-in-oil emulsions, fats,
waxes, oil, silicones, humectants (e.g., glycerol), a
jelly (e.g., SURGILUBE, KY jelly), or a combination
thereof. The formulation may be provided as an
aqueous solution.

30   The formulation preferably does not include an
organic solvent. The formulation may be applied after
the skin has been swabbed with alcohol. However,
removal of the keratinocyte layer prior to application
of the formulation to the extent achieved with a
35 depilatory agent is not preferred.

The antigen may be derived from a pathogen that
can infect the organism (e.g., bacterium, virus,

fungus, or parasite), or a cell (e.g., tumor cell or
normal cell). The antigen may be a tumor antigen or
an autoantigen. Chemically, the antigen may be a
carbohydrate, glycolipid, glycoprotein, lipid,
5   lipoprotein, phospholipid, polypeptide, or chemical or
recombinant conjugate of the above. The molecular
weight of the antigen may be greater than 500 daltons,
preferably greater than 800 daltons, and more
preferably greater than 1000 daltons.
10   Antigen may be obtained by recombinant means,
chemical synthesis, or purification from a natural
source. Preferred are proteinaceous antigen or
conjugates with polysaccharide. Antigen may be at
least partially purified in cell-free form.
15   Alternatively, antigen may be provided in the form of
a live virus, an attenuated live virus, or an
inactivated virus.
         Inclusion of an adjuvant may allow potentiation
or modulation of the immune response. Moreover,
20   selection of a suitable antigen or adjuvant may allow
preferential induction of a humoral or cellular immune
response, specific antibody isotypes (e.g., IgM, IgD,
IgA1, IgA2, IgE, IgG1, IgG2, IgG3, IgG4, or a
combination thereof), and/or specific T-cell subsets
25   (e.g., CTL, Th1, Th2, T_{DTH}, or a combination thereof).
         Preferably, the adjuvant is an ADP-ribosylating
exotoxin or a subunit thereof. Optionally, an
activator of Langerhans cells may be used.
         Optionally, antigen, adjuvant, or both may be
30   provided in the formulation by means of a nucleic acid
(e.g., DNA, RNA, cDNA, cRNA) encoding the antigen or
adjuvant as appropriate. This technique is called
genetic immunization.
         The term "antigen" as used in the invention, is
35   meant to describe a substance that induces a specific
immune response when presented to immune cells of an
organism. An antigen may comprise a single

8

immunogenic epitope, or a multiplicity of immunogenic epitopes recognized by a B-cell receptor (i.e., antibody on the membrane of the B cell) or a T-cell receptor. A molecule may be both an antigen and an

5   adjuvant (e.g., cholera toxin) and, thus, the formulation may contain only one component.

The term "adjuvant" as used in the invention, is meant to describe a substance added to the formulation to assist in inducing an immune response to the

10   antigen. A substance may act as both adjuvant and antigen by inducing both immunostimulation and a specific antibody or T-cell response.

The term "effective amount" as used in the invention, is meant to describe that amount of antigen

15   which induces an antigen-specific immune response. Such induction of an immune response may provide a treatment such as, for example, immunoprotection, desensitization, immunosuppression, modulation of autoimmune disease, potentiation of cancer

20   immunosurveillance, or therapeutic vaccination against an established infectious disease.

The term "draining lymph node field" as used in the invention means an anatomic area over which the lymph collected is filtered through a set of defined

25   set of lymph nodes (e.g., cervical, axillary, inguinal, epitrochelear, popliteal, those of the abdomen and thorax).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows cholera toxin (CT) induces

30   enhanced major histocompatibility complex (MHC) class II expression on Langerhans cells (LC), changes in LC morphology, and loss of LCs (presumably through migration). BALB/c mice (H-2^d) were transcutaneously immunized with 250 μg of cholera CT or its B subunit

35   (CTB) in saline solution on the ear. Previous

.9

experiments had established that mice were readily
immunized when using the skin of the ear (7000 anti-CT
ELISA Units after a single immunization). After 16
hours, epidermal sheets were prepared and stained for
5   MHC class II molecules (scale bar is 50 μm). Panels
indicate (A) saline alone as a negative control, (B)
transcutaneous immunization with CT in saline, (C)
transcutaneous immunization with CTB in saline, and
(D) intradermal injection with tumor necrosis factor-α
10   (10 μg) as a positive control.

DETAILED DESCRIPTION OF THE INVENTION

A transcutaneous immunization system delivers
agents to specialized cells (e.g., antigen
presentation cell, lymphocyte) that produce an immune
15   response (Bos, 1997). These agents as a class are
called antigens. Antigen may be composed of chemicals
such as, for example, carbohydrate, glycolipid,
glycoprotein, lipid, lipoprotein, phospholipid,
polypeptide, protein, conjugates thereof, or any other
20   material known to induce an immune response. Antigen
may be provided as a whole organism such as, for
example, a bacterium or virion; antigen may be
obtained from an extract or lysate, either from whole
cells or membrane alone; or antigen may be chemically
25   synthesized or produced by recombinant means, or by
inactivation of a virus.
Processes for preparing a pharmaceutical
formulation are well-known in the art, whereby the
antigen and adjuvant is combined with a
30   pharmaceutically acceptable carrier vehicle. Suitable
vehicles and their preparation are described, for
example, in Remington's Pharmaceutical Sciences by
E.W. Martin. Such formulations will contain an
effective amount of the antigen and adjuvant together

with a suitable amount of vehicle in order to prepare
pharmaceutically acceptable compositions suitable for
administration to a human or animal. The formulation
may be applied in the form of an cream, emulsion, gel,

5   lotion, ointment, paste, solution, suspension, or
other forms known in the art. In particular,
formulations that enhance skin hydration, penetration,
or both are preferred. There may also be incorporated
other pharmaceutically acceptable additives including,

10   for example, diluents, binders, stabilizers,
preservatives, and colorings.

        Increasing hydration of the stratum corneum will
increase the rate of percutaneous absorbtion of a
given solute (Roberts and Walker, 1993). As used in

15   the present invention, "penetration enhancer" does not
include substances such as, for example: water,
physiological buffers, saline solutions, and alcohols
which would not perforate the skin.

        An object of the present invention is to provide

20   a novel means for immunization through intact skin
without the need for perforating the skin. The
transcutaneous immunization system provides a method
whereby antigens and adjuvant can be delivered to the
immune system, especially specialized antigen

25   presentation cells underlying the skin such as, for
example, Langerhans cells.

        Without being bound to any particular theory but
only to provide an explanation for our observations,
it is presumed that the transcutaneous immunization

30   delivery system carries antigen to cells of the immune
system where an immune response is induced. The
antigen may pass through the normal protective outer
layers of the skin (i.e., stratum corneum) and induce
the immune response directly, or through an antigen

35   presenting cell (e.g., macrophage, tissue macrophage,
Langerhans cell, dendritic cell, dermal dendritic
cell, B lymphocyte, or Kupffer cell) that presents

processed antigen to a T lymphocyte. Optionally, the antigen may pass through the stratum corneum via a hair follicle or a skin organelle (e.g., sweat gland, oil gland).

5   Transcutaneous immunization with bacterial ADP-ribosylating exotoxins (bAREs) may target the epidermal Langerhans cell, known to be among the most efficient of the antigen presenting cells (APCs) (Udey, 1997). We have found that bAREs activate

10 Langerhans cells when applied epicutaneously to the skin in saline solution. The Langerhans cells direct specific immune responses through phagocytosis of the antigens, and migration to the lymph nodes where they act as APCs to present the antigen to lymphocytes

15 (Udey, 1997), and thereby induce a potent antibody response. Although the skin is generally considered a barrier to invading organisms, the imperfection of this barrier is attested to by the numerous Langerhans cells distributed throughout the epidermis that are

20 designed to orchestrate the immune response against organisms invading via the skin (Udey, 1997).

According to Udey (1997):

"Langerhans cells are bone-marrow
derived cells that are present in all
25   mammalian stratified squamous epithelia.
They comprise all of the accessory cell
activity that is present in uninflammed
epidermis, an in the current paradigm are
essential for the initiation and propagation
30   of immune responses directed against
epicutaneously applied antigens. Langerhans
cells are members of a family of potent
accessory cells ('dendritic cells') that are
widely distributed, but infrequently
35   represented, in epithelia and solid organs
as well as in lymphoid tissue . . .
"It is now recognized that Langerhans
cells (and presumably other dendritic cells)
have a life cycle with at least two distinct
40   stages. Langerhans cells that are located
in epidermis constitute a regular network of
antigen-trapping 'sentinel' cells.
Epidermal Langerhans cells can ingest
particulates, including microorganisms, and

12

are efficient processors of complex
antigens. However, they express only low
levels of MHC class I and II antigens and
costimulatory molecules (ICAM-1, B7-1 and
5   B7-2) and are poor stimulators of unprimed T
cells. After contact with antigen, some
Langerhans cells become activated, exit the
epidermis and migrate to T-cell-dependent
regions of regional lymph nodes where they
10   local as mature dendritic cells. In the
course of exiting the epidermis and
migrating to lymph nodes, antigen-bearing
epidermal Langerhans cells (now the
'messengers') exhibit dramatic changes in
15   morphology, surface phenotype and function.
In contrast to epidermal Langerhans cells,
lymphoid dendritic cells are essentially
non-phagocytic and process protein antigens
inefficiently, but express high levels of
20   MHC class I and class II antigens and
various costimulatory molecules and are the
most potent stimulators of naive T cells
that have been identified."

We envision that the potent antigen presenting
25   capability of the epidermal Langerhans cells can be
exploited for transcutaneously delivered vaccines. A
transcutaneous immune response using the skin immune
system would require delivery of vaccine antigen only
to Langerhans cells in the stratum corneum (the
30   outermost layer of the skin consisting of cornified
cells and lipids) via passive diffusion and subsequent
activation of the Langerhans cells to take up antigen,
migrate to B-cell follicles and/or T-cell dependent
regions, and present the antigen to B and/or T cells
35   (Stingl et al., 1989). If antigens other that bAREs
(for example BSA) were to be phagocytosed by the
Langerhans cells, then these antigens could also be
taken to the lymph node for presentation to T-cells
and subsequently induce an immune response specific
40   for that antigen (e.g., BSA). Thus, a feature of
transcutaneous immunization is the activation of the
Langerhans cell, presumably by a bacterial ADP-
ribosylating exotoxin, ADP-ribosylating exotoxin
binding subunits (e.g., cholera toxin B subunit), or

13

other Langerhans cell activating substance.

The mechanism of transcutaneous immunization via Langerhans cells activation, migration and antigen presentation is clearly shown by the upregulation of
5   MHC class II expression in the epidermal Langerhans cells from epidermal sheets transcutaneously immunized with CT or CTB. In addition, the magnitude of the antibody response induced by transcutaneous immunization and isotype switching to predominantly
10   IgG is generally achieved with T-cell help stimulated by antigen presenting cells such as Langerhans cells or dendritic cells (Janeway and Travers, 1996), and activation of both Th1 and Th2 pathways as suggested by the production of IgG1 and IgG2a (Paul and Seder,
15   1994; Seder and Paul, 1994). Additionally, T cell proliferation to the antigen OVA is shown in mice immunized with CT + OVA. Alternatively, a large antibody response may be induced by a thymus-independent antigen type 1 (TI-1) which directly
20   activates the B cell (Janeway and Travers, 1996).

The spectrum of more commonly known skin immune responses is represented by contact dermatitis and atopy. Contact dermatitis, a pathogenic manifestation of LC activation, is directed by Langerhans cells
25   which phagocytose antigen, migrate to lymph nodes, present antigen, and sensitize T cells for the intense destructive cellular response that occurs at the affected skin site (Dahl, 1996; Leung, 1997). Atopic dermatitis may utilize the Langerhans cell in a
30   similar fashion, but is identified with Th2 cells and is generally associated with high levels of IgE, antibody (Dahl, 1996; Leung, 1997).

Transcutaneous immunization with cholera toxin and related bAREs on the other hand is a novel immune
35   response with an absence of superficial and microscopic post-immunization skin findings (i.e., non-inflamed skin) shown by the absence of lymphocyte

·14

infiltration 24, 48 and 120 hours after immunization
with cholera toxin. This indicates that Langerhans
cells "comprise all of the accessory cell activity
that is present in uninflammed epidermis, and in the
5   current paradigm are essential for the initiation and
propagation of immune responses directed against
epicutaneously applied antigens" (Udey, 1997). The
uniqueness of the transcutaneous immune response here
is also indicated by the both high levels of antigen-
10   specific IgG antibody, and the type of antibody
produced (e.g., IgM, IgG1, IgG2a, IgG2b, IgG3 and IgA)
and the absence of anti-CT IgE antibody.
        Thus, we have found that bacterial-derived toxins
applied to the surface of the skin can activate
15   Langerhans cells or other antigen presenting cells,
and induce a potent immune response manifested as high
levels of antigen-specific circulating IgG antibodies.
Such adjuvants may be used in transcutaneous
immunization to enhance the IgG antibody response to
20   proteins not otherwise immunogenic by themselves when
placed on the skin.
        Transcutaneous targeting of Langerhans cells may
also be used to deactivate their antigen presenting
function, thereby preventing immunization or
25   sensitization. Techniques to deactivate Langerhans
cells include, for example, the use of interleukin-10
(Peguet-Navarro et al., 1995), monoclonal antibody to
interleukin-1β (Enk et al., 1993), or depletion via
superantigens such as through staphylococcal
30   enterotoxin-A (SEA) induced epidermal Langerhans cell
depletion (Shankar et al., 1996).
        Transcutaneous immunization may be induced via
the ganglioside GM1 binding activity of CT, LT or
subunits such as CTB (Craig and Cuatrecasas, 1975).
35   Ganglioside GM1 is a ubiquitous cell membrane
glycolipid found in all mammalian cells (Plotkin and
Mortimer, 1994). When the pentameric CT B subunit

15

binds to the cell surface a hydrophilic pore is formed
which allows the A subunit to penetrate across the
lipid bilayer (Ribi et al., 1988).

　　　　We have shown that transcutaneous immunization by
5　　CT or CTB may require ganglioside GM1 binding
activity. When mice were transcutaneously immunized
with CT, CTA and CTB, only CT and CTB resulted in an
immune response. CTA contains the ADP-ribosylating
exotoxin activity but only CT and CTB containing the
10　binding activity were able to induce an immune
response indicating that the B subunit was necessary
and sufficient to immunize through the skin. We
conclude that the Langerhans cell or another antigen
presenting cell may be activated by CTB binding to its
15　cell surface.

ANTIGEN
　　　　Antigen of the invention may be expressed by
recombinant means, preferably as a fusion with an
affinity or epitope tag (Summers and Smith, 1987;
20　Goeddel, 1990; Ausubel et al., 1996); chemical
synthesis of an oligopeptide, either free or
conjugated to carrier proteins, may be used to obtain
antigen of the invention (Bodanszky, 1993; Wisdom,
1994). Oligopeptides are considered a type of
25　polypeptide.
　　　　Oligopeptide lengths of 6 residues to 20 residues
are preferred. Polypeptides may also by synthesized
as branched structures such as those disclosed in U.S.
Pat. Nos. 5,229,490 and 5,390,111. Antigenic
30　polypeptides include, for example, synthetic or
recombinant B-cell and T-cell epitopes, universal T-
cell epitopes, and mixed T-cell epitopes from one
organism or disease and B-cell epitopes from another.
　　　　Antigen obtained through recombinant means or
35　peptide synthesis, as well as antigen of the invention
obtained from natural sources or extracts, may be

16

purified by means of the antigen's physical and
chemical characteristics, preferably by fractionation
or chromatography (Janson and Ryden, 1989; Deutscher,
1990; Scopes, 1993).

5   A multivalent antigen formulation may be used to
induce an immune response to more than one antigen at
the same time. Conjugates may be used to induce an
immune response to multiple antigens, to boost the
immune response, or both. Additionally, toxins may be
10 boosted by the use of toxoids, or toxoids boosted by
the use of toxins. Transcutaneous immunization may be
used to boost responses induced initially by other
routes of immunization such as by injection, or the
oral or intranasal routes.

15   Antigen includes, for example, toxins, toxoids,
subunits thereof, or combinations thereof (e.g.,
cholera toxin, tetanus toxoid).

Antigen may be solubilized in water, a solvent
such as methanol, or a buffer. Suitable buffers
20 include, but are not limited to, phosphate buffered
saline Ca^{++}/Mg^{++} free (PBS), normal saline (150 mM NaCl
in water), and Tris buffer. Antigen not soluble in
neutral buffer can be solubilized in 10 mM acetic acid
and then diluted to the desired volume with a neutral
25 buffer such as PBS. In the case of antigen soluble
only at acid pH, acetate-PBS at acid pH may be used as
a diluent after solubilization in dilute acetic acid.
Glycerol may be a suitable non-aqueous buffer for use
in the present invention.

30   If an antigen such as, for example, hepatitis A
virus, is not soluble per se, the antigen may be
present in the formulation in a suspension or even as
an aggregate.

Hydrophobic antigen can be solubilized in a
35 detergent, for example a polypeptide containing a
membrane-spanning domain. Furthermore, for
formulations containing liposomes, an antigen in a

17

detergent solution (e.g., a cell membrane extract) may
be mixed with lipids, and liposomes then may be formed
by removal of the detergent by dilution, dialysis, or
column chromatography. Certain antigens such as, for
5   example, those from a virus (e.g., hepatitis A) need
not be soluble per se, but can be incorporated
directly into a liposome in the form of a virosome
(Morein and Simons, 1985).

Plotkin and Mortimer (1994) provide antigens
10   which can be used to vaccinate animals or humans to
induce an immune response specific for particular
pathogens, as well as methods of preparing antigen,
determining a suitable dose of antigen, assaying for
induction of an immune response, and treating
15   infection by a pathogen (e.g., bacterium, virus,
fungus, or parasite).

Bacteria include, for example: anthrax,
campylobacter, cholera, diphtheria, enterotoxigenic *E.
coli*, giardia, gonococcus, *Helicobacter pylori* (Lee
20   and Chen, 1994), *Hemophilus influenza* B, *Hemophilus
influenza* non-typable, meningococcus, pertussis,
pneumococcus, salmonella, shigella, *Streptococcus* B,
group A *Streptococcus*, tetanus, *Vibrio cholerae*,
yersinia, *Staphylococcus*, *Pseudomonas* species and
25   *Clostridia* species.

Viruses include, for example: adenovirus, dengue
serotypes 1 to 4 (Delenda et al., 1994; Fonseca et
al., 1994; Smucny et al., 1995), ebola (Jahrling et
al., 1996), enterovirus, hepatitis serotypes A to E
30   (Blum, 1995; Katkov, 1996; Lieberman and Greenberg,
1996; Mast, 1996; Shafara et al., 1995; Smedila et
al., 1994; U.S. Pat. Nos. 5,314,808 and 5,436,126),
herpes simplex virus 1 or 2, human immunodeficiency
virus (Deprez et al., 1996), influenza, Japanese
35   equine encephalitis, measles, Norwalk, papilloma
virus, parvovirus B19, polio, rabies, rotavirus,
rubella, rubeola, vaccinia, vaccinia constructs

18

containing genes coding for other antigens such as
malaria antigens, varicella, and yellow fever.

Parasites include, for example: *Entamoeba
histolytica* (Zhang et al., 1995); *Plasmodium* (Bathurst
5   et al., 1993; Chang et al., 1989, 1992, 1994; Fries et
al., 1992a, 1992b; Herrington et al., 1991; Khusmith
et al., 1991; Malik et al., 1991; Migliorini et al.,
1993; Pessi et al., 1991; Tam, 1988; Vreden et al.,
1991; White et al., 1993; Wiesmueller et al., 1991),
10   *Leishmania* (Frankenburg et al., 1996), Toxoplasmosis,
and the Helminths.

Antigens may also comprise those used in
biological warfare such as ricin, for which protection
can be achieved via antibodies.

15   ADJUVANT
The formulation also contains an adjuvant,
although a single molecule may contain both adjuvant
and antigen properties (e.g., cholera toxin) (Elson
and Dertzbaugh, 1994). Adjuvants are substances that
20   are used to specifically or non-specifically
potentiate an antigen-specific immune response.
Usually, the adjuvant and the formulation are mixed
prior to presentation of the antigen but,
alternatively, they may be separately presented within
25   a short interval of time.
Adjuvants include, for example, an oil emulsion
(e.g., complete or incomplete Freund's adjuvant), a
chemokine (e.g., defensins 1 or 2, RANTES, MIP1-α,
MIP-2, interleukin-8) or a cytokine (e.g.,
30   interleukin-1β, -2, -6, -10 or -12; γ-interferon; tumor
necrosis factor-α; or granulocyte-monocyte-colony
stimulating factor) (reviewed in Nohria and Rubin,
1994), a muramyl dipeptide derivative (e.g.,
murabutide, threonyl-MDP or muramyl tripeptide), a
35   heat shock protein or a derivative, a derivative of

Leishmania major LeIF (Skeiky et al., 1995), cholera
toxin or cholera toxin B, a lipopolysaccharide (LPS)
derivative (e.g., lipid A or monophosphoryl lipid A),
or superantigen (Saloga et al., 1996). Also, see

5   Richards et al. (1995) for adjuvants useful in
immunization.

An adjuvant may be chosen to preferentially
induce antibody or cellular effectors, specific
antibody isotypes (e.g., IgM, IgD, IgA1, IgA2,

10   secretory IgA, IgE, IgG1, IgG2, IgG3, and/or IgG4), or
specific T-cell subsets (e.g., CTL, Th1, Th2 and/or
T_{DTH}) (Glenn et al., 1995).

Cholera toxin is a bacterial exotoxin from the
family of ADP-ribsoylating exotoxins (referred to as

15   bAREs). Most bAREs are organized as A:B dimer with a
binding B subunit and an A subunit containing the ADP-
ribosyltransferase. Such toxins include diphtheria
toxin, *Pseudomonas* exotoxin A, cholera toxin (CT), *E.
coli* heat-labile enterotoxin (LT), pertussis toxin, *C.*

20   *botulinum* toxin C2, *C. botulinum* toxin C3, *C. limosum*
exoenzyme, *B. cereus* exoenzyme, *Pseudomonas* exotoxin
S, *Staphylococcus aureus* EDIN, and *B. sphaericus*
toxin.

Cholera toxin is an example of a bARE that is

25   organized with A and B subunits. The B subunit is the
binding subunit and consists of a B-subunit pentamer
which is non-covalently bound to the A subunit. The
B-subunit pentamer is arranged in a symmetrical
doughnut-shaped structure that binds to GM_1-ganglioside

30   on the target cell. The A subunit serves to ADP
ribosylate the alpha subunit of a subset of the hetero
trimeric GTP proteins (G proteins) including the Gs
protein which results in the elevated intracellular
levels of cyclic AMP. This stimulates release of ions

35   and fluid from intestinal cells in the case of
cholera.

Cholera toxin (CT) and its B subunit (CTB) have

20

adjuvant properties when used as either an
intramuscular or oral immunogen (Elson and Dertzbaugh,
1994; Trach et al., 1997). Another antigen, heat-
labile enterotoxin from *E. coli* (LT) is 80% homologous

5   at the amino acid level with CT and possesses similar
binding properties; it also appears to bind the GM_1-
ganglioside receptor in the gut and has similar ADP-
ribosylating exotoxin activities. Another bARE,
Pseudomonas exotoxin A (ETA), binds to the α_2-

10   macroglobulin receptor-low density lipoprotein
receptor-related protein (Kounnas et al., 1992).
bAREs are reviewed by Krueger and Barbieri (1995).
        The toxicity of CT by oral, nasal, and
intramuscular routes limits the dose that can be used

15   as an adjuvant. In a comparative trial of CT injected
intramuscularly, extensive swelling at the site of
injection was elicited. By contrast, equivalent or
greater doses of CT placed on the skin caused no
toxicity.

20   The examples below show that cholera toxin (CT),
its B subunit (CTB), *E. coli* heat-labile enterotoxin
(LT), and pertussis toxin are potent adjuvants for
transcutaneous immunization, inducing high levels of
IgG antibodies but not IgE antibodies. Also shown is

25   that CTB without CT can also induce high levels of IgG
antibodies. Thus, both bAREs and a derivative thereof
can effectively immunize when epicutaneouly applied to
the skin in a simple solution. Furthermore, these
examples demonstrate that CT, CTB and bAREs can act as

30   both adjuvant and antigen.
        When an adjuvant such as CT is mixed with BSA, a
protein not usually immunogenic when applied to the
skin, anti-BSA antibodies are induced. An immune
response to diphtheria toxoid was induced using

35   pertussis toxin as adjuvant, but not with diphtheria
toxoid alone. Thus, bAREs can act as adjuvants for
non-immunogenic proteins in an transcutaneous

21

immunization system.

Other proteins may also act as both adjuvant and antigen. For example FLUZONE (Lederle), the split virion influenza A and B vaccine contains

5   neuraminidase and hemagglutinin which are highly immunogenic, confers protection and may effectively immunize through the skin acting as its own adjuvant and antigen. Toxoids such as diphtheria toxoid which has been toxoided using formalin, pertussis toxoid

10   which has been toxoided using hydrogen peroxide, or mutant toxins such as cholera or heat labile enterotoxin from _E. coli_ which have been toxoided using genetic techniques to destroy the ribosyl transferase activity, may continue to harbor adjuvant

15   qualities and act as both antigen and adjuvant.

Protection against the life-threatening infections diphtheria, pertussis, and tetanus (DPT) can be achieved by inducing high levels of circulating anti-toxin antibodies. Pertussis may be an exception

20   in that some investigators feel that antibodies directed to other portions of the invading organism are necessary for protection, although this is controversial (see Schneerson et al., 1996) and most new generation acellular pertussis vaccines have PT as

25   a component of the vaccine (Krueger and Barbieri, 1995). The pathologies in the diseases caused by DPT are directly related to the effects of their toxins and anti-toxin antibodies most certainly play a role in protection (Schneerson et al., 1996).

30   In general, toxins can be chemically inactivated to form toxoids which are less toxic but remain immunogenic. We envision that the transcutaneous immunization system using toxin-based immunogens and adjuvants can achieve anti-toxin levels adequate for

35   protection against these diseases. The anti-toxin antibodies may be induced through immunization with the toxins, or genetically-detoxified toxoids

themselves, or with toxoids and adjuvants such as CT
or by the toxoids alone. Genetically toxoided toxins
which have altered ADP-ribosylating exotoxin activity,
but not binding activity, are envisioned to be

5   especially useful as non-toxic activators of antigen
presenting cells used in transcutaneous immunization.
    We envision that CT can also act as an adjuvant
to induce antigen-specific CTLs through transcutaneous
immunization (see Bowen et al., 1994; Porgador et al.,

10   1997 for the use of CT as an adjuvant in oral
immunization).
    The bARE adjuvant may be chemically conjugated to
other antigens including, for example, carbohydrates,
polypeptides, glycolipids, and glycoprotein antigens.

15   Chemical conjugation with toxins, their subunits, or
toxoids with these antigens would be expected to
enhance the immune response to these antigens when
applied epicutaneously.
    To overcome the problem of the toxicity of the

20   toxins, (e.g., diphtheria toxin is known to be so
toxic that one molecule can kill a cell) and to
overcome the difficulty of working with such potent
toxins as tetanus, several workers have taken a
recombinant approach to producing genetically produced

25   toxoids. This is based on inactivating the catalytic
activity of the ADP-ribosyl transferase by genetic
deletion. These toxins retain the binding
capabilities, but lack the toxicity, of the natural
toxins. This approach is described by Burnette et al.

30   (1994), Rappuoli et al. (1995), and Rappuoli et al.
(1996). Such genetically toxoided exotoxins could be
useful for transcutaneous immunization system in that
they would not create a safety concern as the toxoids
would not be considered toxic. They may act as both

35   antigens and adjuvants, enhancing the immune response
to themselves or added antigens. Additionally,
several techniques exist to chemically toxoid toxins

23

which can address the same problem (Schneerson et al.,
1996). Alternatively, fragments of the toxin or
toxoids may be used such as the C fragment of Tetanus.
These techniques could be important for certain
5   applications, especially pediatric applications, in
which ingested toxins (e.g., diphtheria toxin) might
possibly create adverse reactions.

Optionally, an activator of Langerhans cells may
be used as an adjuvant. Examples of such activators
10   include: inducers of heat shock protein; contact
sensitizers (e.g., trinitrochlorobenzene, dinitro-
fluorobenzene, nitrogen mustard, pentadecylcatechol);
toxins (e.g, Shiga toxin, Staph enterotoxin B); lipo-
polysaccharides, lipid A, or derivatives thereof;
15   bacterial DNA (Stacey et al., 1996); cytokines (e.g.,
tumor necrosis factor-α, interleukin-1β, -10, -12);
and chemokines (e.g., defensins 1 or 2, RANTES, MIP-
1α, MIP-2, interleukin-8).

A combination of different adjuvants may be used
20   in the present invention. For example, a combination
of bacterial DNA containing CpG nucleotide sequences
and an ADP-ribosylating exotoxin could be used to
direct the T-helper response to antigens administered
transcutaneously. Thus, Th1 or Th2 like responses to
25   CT-adjuvanted antigens could be switched by the use of
nonmethylated CpG bacterial DNA, or other proteins
such as LeIF or calcium channel blockers.

CpGs are among a class of structures which have
patterns allowing the immune system to recognize their
30   pathogenic origins to stimulate the innate immune
response leading to adaptive immune responses.
(Medzhitov and Janeway, Curr. Opin. Immunol., 9:4-9,
1997). These structures are called pathogen-
associated molecular patterns (PAMPs) and include
35   lipopolysaccharides, teichoic acids, unmethylated CpG
motifs, double stranded RNA and mannins.

24

PAMPs induce endogenous signals that can mediate
the inflammatory response, act as costimulators of T-
cell function and control the effector function. The
ability of PAMPs to induce these responses play a role

5   in their potential as adjuvants and their targets are
APCs such as macrophages and dendritic cells. The
antigen presenting cells of the skin could likewise be
stimulated by PAMPs transmitted through the skin. For
example, Langerhans cells, a type of dendritic cell,

10   could be activated by a PAMP in solution on the skin
with a transcutaneously poorly immunogenic molecule
and be induced to migrate and present this poorly
immunogenic molecule to T-cells in the lymph node,
inducing an antibody response to the poorly

15   immunogenic molecule. PAMPs could also be used in
conjunction with other skin adjuvants such as cholera
toixn to induce different costimulatory molecules and
control different effector functions to guide the
immune response, for example from a Th2 to a Th1

20   response.
      If an immunizing antigen has sufficient
Langerhans cell activating capabilities then a
separate adjuvant may not be required, as in the case
of CT which is both antigen and adjuvant. It is

25   envisioned that whole cell preparations, live viruses,
attenuated viruses, DNA plasmids, and bacterial DNA
could be sufficient to immunize transcutaneously. It
may be possible to use low concentrations of contact
sensitizers or other activators of Langerhans cells to

30   induce an immune response without inducing skin
lesions.

LIPOSOMES AND THEIR PREPARATION
      Liposomes are closed vesicles surrounding an

35   internal aqueous space. The internal compartment is
separated from the external medium by a lipid bilayer
composed of discrete lipid molecules. In the present

25

invention, antigen may be delivered through intact
skin to specialized cells of the immune system,
whereby an antigen-specific immune response is
induced. Transcutaneous immunization may be achieved
5   by using liposomes; however, as shown in the examples,
liposomes are not required to elicit an antigen-
specific immune response.

     Liposomes may be prepared using a variety of
techniques and membrane lipids (reviewed in
10 Gregoriadis, 1993). Liposomes may be pre-formed and
then mixed with antigen. The antigen may be dissolved
or suspended, and then added to (a) the pre-formed
liposomes in a lyophilized state, (b) dried lipids as
a swelling solution or suspension, or (c) the solution
15 of lipids used to form liposomes. They may also be
formed from lipids extracted from the stratum corneum
including, for example, ceramide and cholesterol
derivatives (Wertz, 1992).

     Chloroform is a preferred solvent for lipids, but
20 it may deteriorate upon storage. Therefore, at one-
to three-month intervals, chloroform is redistilled
prior to its use as the solvent in forming liposomes.
After distillation, 0.7% ethanol can be added as a
preservative. Ethanol and methanol are other suitable
25 solvents.

     The lipid solution used to form liposomes is
placed in a round-bottomed flask. Pear-shaped boiling
flasks are preferred, particularly those flasks sold
by Lurex Scientific (Vineland, NJ, cat. no. JM-5490).
30 The volume of the flask should be more than ten times
greater than the volume of the anticipated aqueous
suspension of liposomes to allow for proper agitation
during liposome formation.

     Using a rotary evaporator, solvent is removed at
35 37°C under negative pressure for 10 minutes with a
filter aspirator attached to a water faucet. The
flask is further dried under low vacuum (i.e., less

26

than 50 mm Hg) for 1 hour in a dessicator.

To encapsulate antigen into liposomes, an aqueous
solution containing antigen may be added to
lyophilized liposome lipids in a volume that results
5   in a concentration of approximately 200 mM with
respect to liposome lipid, and shaken or vortexed
until all the dried liposome lipids are wet. The
liposome-antigen mixture may then be incubated for 18
hours to 72 hours at 4°C. The liposome-antigen
10 formulation may be used immediately or stored for
several years. It is preferred to employ such a
formulation directly in the transcutaneous
immunization system without removing unencapsulated
antigen. Techniques such as bath sonication may be
15 employed to decrease the size of liposomes, which may
augment transcutaneous immunization.

Liposomes may be formed as described above but
without addition of antigen to the aqueous solution.
Antigen may then be added to the pre-formed liposomes
20 and, therefore, antigen would be in solution and/or
associated with, but not encapsulated by, the
liposomes. This process of making a liposome-
containing formulation is preferred because of its
simplicity. Techniques such as bath sonication may be
25 employed to alter the size and/or lamellarity of the
liposomes to enhance immunization.

Although not required to practice the present
invention, hydration of the stratum corneum may be
enhanced by adding liposomes to the formulation.
30 Liposomes have been used as carriers with adjuvants to
enhance the immune response to antigens mixed with,
encapsulated in, attached to, or associated with
liposomes.

35 TRANSCUTANEOUS DELIVERY OF ANTIGEN

Efficient immunization can be achieved with the
present invention because transcutaneous delivery of

27

antigen may target the Langerhans cell. These cells
are found in abundance in the skin and are efficient
antigen presenting cells leading to T-cell memory and
potent immune responses (Udey, 1997). Because of the
5   presence of large numbers of Langerhans cells in the
skin, the efficiency of transcutaneous delivery may be
related to the surface area exposed to antigen and
adjuvant. In fact, the reason that transcutaneous
immunization is so effective may be that it targets a
10   larger number of these efficient antigen presenting
cells than intramuscular immunization. However, even
a small number of Langerhans cells or dendritic cells
may be sufficient for immunization.
      We envision the present invention will enhance
15   access to immunization, while inducing a potent immune
response. Because transcutaneous immunization does
not involve penetration of the skin and the
complications and difficulties thereof, the
requirements of trained personnel, sterile technique,
20   and sterile equipment are reduced. Furthermore, the
barriers to immunization at multiple sites or to
multiple immunizations are diminished. Immunization
by a single application of the formulation is also
envisioned.
25   Immunization may be achieved using epicutaneous
application of a simple solution of antigen and
adjuvant impregnated in gauze under an occlusive
patch, or by using other patch technologies; creams,
immersion, ointments and sprays are other possible
30   methods of application. The immunization could be
given by untrained personnel, and is amenable to self-
application. Large-scale field immunization could
occur given the easy accessibility to immunization.
Additionally, a simple immunization procedure would
35   improve access to immunization by pediatric patients
and the elderly, and populations in Third World
countries.

28

Similarly, animals could be immunized using the
present invention. Application to anatomical sites
such as the ear, underbelly, paws, conjunctiva,
intertriginous regions, or anal region, or via dipping
5   or immersion could be employed.

For previous vaccines, their formulations were
injected through the skin with needles. Injection of
vaccines using needles carries certain drawbacks
including the pain associated with injections, the
10   need for sterile needles and syringes, trained medical
personnel to administer the vaccine, discomfort from
the injection, and potential complications brought
about by puncturing the skin with the needle.
Immunization through the skin without the use of
15   needles (i.e., transcutaneous immunization) represents
a major advance for vaccine delivery by avoiding the
aforementioned drawbacks.

The transcutaneous delivery system of the
invention is also not concerned with penetration of
20   intact skin by sound or electrical energy. Such a
system that uses an electrical field to induce
dielectric breakdown of the stratum corneum is
disclosed in U.S. Pat. No. 5,464,386.

Moreover, transcutaneous immunization may be
25   superior to immunization using needles as more immune
cells would be targeted by the use of several
locations targeting large surface areas of skin. A
therapeutically effective amount of antigen sufficient
to induce an immune response may be delivered
30   transcutaneously either at a single cutaneous
location, or over an area of intact skin covering
multiple draining lymph node fields (e.g., cervical,
axillary, inguinal, epitrochelear, popliteal, those of
the abdomen and thorax). Such locations close to
35   numerous different lymphatic nodes at locations all
over the body will provide a more widespread stimulus
to the immune system than when a small amount of

29

antigen is injected at a single location by
intradermal subcutaneous or intramuscular injection.

Antigen passing through or into the skin may
encounter antigen presenting cells which process the
5   antigen in a way that induces an immune response.
Multiple immunization sites may recruit a greater
number of antigen presenting cells and the larger
population of antigen presenting cells that were
recruited would result in greater induction of the
10   immune response. Transcutaneous immunization may
allow application in close proximity to a lymph node
draining site and thereby improve efficiency or
potency of immunization. It is conceivable that
absorption through the skin may deliver antigen to
15   phagocytic cells of the skin such as, for example,
dermal dendritic cells, macrophages, and other skin
antigen presenting cells; antigen may also be
delivered to phagocytic cells of the liver, spleen,
and bone marrow that are known to serve as the antigen
20   presenting cells through the blood stream or lymphatic
system. The result would be widespread distribution
of antigen to antigen presenting cells to a degree
that is rarely, if ever achieved, by current
immunization practices.
25   The transcutaneous immunization system may be
applied directly to the skin and allowed to air dry;
rubbed into the skin or scalp; held in place with a
dressing, patch, or absorbent material; otherwise held
by a device such as a stocking, slipper, glove, or
30   shirt; or sprayed onto the skin to maximize contact
with the skin. The formulation may be applied in an
absorbant dressing or gauze. The formulation may be
covered with an occlusive dressing such as, for
example, in an emulsion of antigen solution and
35   AQUAPHOR (petrolatum, mineral oil, mineral wax, wool
wax, panthenol, bisabol, and glycerin from
Beiersdorf), plastic film, impregnated polymer,

30

COMFEEL (Coloplast) or vaseline; or a non-occlusive
dressing such as, for example, DUODERM (3M) or OPSITE
(Smith & Napheu). An occlusive dressing completely
excludes the passage of water. Alternatively, a
5   partially occlusive dressing such as TEGADERM may be
applied to provide hydration and may allow longer
application of the patch or may prevent maceration of
the skin.

      The formulation may be applied to single or
10   multiple sites, to single or multiple limbs, or to
large surface areas of the skin by complete immersion.
The formulation may be applied directly to the skin.

      Genetic immunization has been described in U.S.
Pat. Nos. 5,589,466 and 5,593,972. The nucleic
15   acid(s) contained in the formulation may encode the
antigen, the adjuvant, or both. The nucleic acid may
or may not be capable of replication; it may be non-
integrating and non-infectious. The nucleic acid may
further comprise a regulatory region (e.g., promoter,
20   enhancer, silencer, transcription initiation and
termination sites, RNA splice acceptor and donor
sites, polyadenylation signal, internal ribosome
binding site, translation initiation and termination
sites) operably linked to the sequence encoding the
25   antigen or adjuvant. The nucleic acid may be
complexed with an agent that promotes transfection
such as cationic lipid, calcium phosphate, DEAE-
dextran, polybrene-DMSO, or a combination thereof.
The nucleic acid may comprise regions derived from
30   viral genomes. Such materials and techniques are
described by Kriegler (1990) and Murray (1991).

      An immune response may comprise humoral (i.e.,
antigen-specific antibody) and/or cellular (i.e.,
antigen-specific lymphocytes such as B cells, CD4$^+$ T
35   cells, CD8$^+$ T cells, CTL, Th1 cells, Th2 cells, and/or
T$_{DTH}$ cells) effector arms. Moreover, the immune
response may comprise NK cells that mediate antibody-

31

dependent cell-mediated cytotoxicity (ADCC).

The immune response induced by the formulation of
the invention may include the elicitation of antigen-
specific antibodies and/or cytotoxic lymphocytes (CTL,
5   reviewed in Alving and Wassef, 1994). Antibody can be
detected by immunoassay techniques, and the detection
of various isotypes (e.g., IgM, IgD, IgA1, IgA2,
secretory IgA, IgE, IgG1, IgG2, IgG3, or IgG4) may be
expected. An immune response can also be detected by
10   a neutralizing assay.

Antibodies are protective proteins produced by B
lymphocytes. They are highly specific, generally
targeting one epitope of an antigen. Often,
antibodies play a role in protection against disease
15   by specifically reacting with antigens derived from
the pathogens causing the disease. Immunization may
induce antibodies specific for the immunizing antigen,
such as cholera toxin. These antigen-specific
antibodies are induced when antigen is delivered
20   through the skin by liposomes.

CTLs are particular protective immune cells
produced to protect against infection by a pathogen.
They are also highly specific. Immunization may
induce CTLs specific for the antigen, such as a
25   synthetic oligopeptide based on a malaria protein, in
association with self-major histocompatibility
antigen. CTLs induced by immunization with the
transcutaneous delivery system may kill pathogen
infected cells. Immunization may also produce a
30   memory response as indicated by boosting responses in
antibodies and CTLs, lymphocyte proliferation by
culture of lymphocytes stimulated with the antigen,
and delayed type hypersensitivity responses to
intradermal skin challenge of the antigen alone.

35   It is envisioned that the T-helper response
induced by transcutaneous immunization may be
manipulated by the use of calcium channel blockers

32

(e.g., nifedipine, verpamil) which suppress the
contact hypersensitivity reaction by inhibiting
antigen catabolism and subsequent presentation by
epidermal Langerhans cells. The transcutaneous
5   application of a calcium channel blocker would be
expected to affect surface expression of co-
stimulatory molecules (e.g., B7-related family) and
the generation of a subsequent T-helper response. It
is also envisioned that addition of the calcium
10   channel blocker may inhibit delayed type
hypersensitivity responses and could be used to select
an immune response that is predominantly a cellular or
a humoral response.

        In a viral neutralization assay, serial dilutions
15   of sera are added to host cells which are then
observed for infection after challenge with infectious
virus. Alternatively, serial dilutions of sera may be
incubated with infectious titers of virus prior to
inoculation of an animal, and the inoculated animals
20   are then observed for signs of infection.

        The transcutaneous immunization system of the
invention may be evaluated using challenge models in
either animals or humans, which evaluate the ability
of immunization with the antigen to protect the
25   subject from disease. Such protection would
demonstrate an antigen-specific immune response. In
lieu of challenge, achieving anti-diphtheria antibody
titers of 5 IU/ml or greater is generally assumed to
indicate optimum protection and serves as a surrogate
30   marker for protection (Plotkin and Mortimer, 1994).

        Furthermore, the *Plasmodium faciparum* challenge
model may be used as to induce an antigen-specific
immune response in humans. Human volunteers may be
immunized using the transcutaneous immunization system
35   containing oligopeptides or proteins (polypeptides)
derived from the malaria parasite, and then exposed to
malaria experimentally or in the natural setting. The

33

Plasmodium yoelii mouse malaria challenge model may be
used to evaluate protection in the mouse against
malaria (Wang et al, 1995).

5   Alving et al (1986) injected liposomes comprising
lipid A as an adjuvant for inducing an immune response
to cholera toxin (CT) in rabbits and to a synthetic
protein consisting of a malaria oligopeptide
containing four tetra-peptides (Asn-Ala-Asn-Pro)
conjugated to BSA. The authors found that the immune
10   response to cholera toxin or to the synthetic malaria
protein was markedly enhanced by encapsulating the
antigen within the liposomes containing lipid A,
compared to similar liposomes lacking lipid A.
Several antigens derived either from the
15   circumsporozoite protein (CSP) or from merozoite
surface proteins of *Plasmodium falciparum* have been
encapsulated in liposomes containing lipid A. All of
the malaria antigens that have been encapsulated in
liposomes containing lipid A have been shown to induce
20   humoral effectors (i.e., antigen-specific antibodies),
and some have been shown to induce cell-mediated
responses as well. Generation of an immune response
and immunoprotection in an animal vaccinated with a
malaria antigen may be assayed by immunofluorescence
25   to whole, fixed malaria sporozoites or CTLs killing of
target cells transfected with CSP.

Mice transcutaneously immunized with cholera
toxin can be protected against intranasal challenge
with a 20 µg dose of cholera toxin. Mallet et al
30   (personal communication) have found that C57B1/6 mice
develop a fatal hemorrhagic pneumonia in response to
intranasal challenge with CT. Alternatively, the mice
may be challenged with an intraperitoneal dose of CT
(Dragunsky et al, 1992). Cholera toxin-specific IgG
35   or IgA antibody may provide protection against cholera
toxin challenge (Pierce, 1978; Pierce and Reynolds,
1974).

34

Similar protective effects could be expected in
humans immunized with LT or CT, and challenged with
LT-secreting *E. coli* or CT-secreting *Vibrio cholerae*,
respectively. Additionally, cross protection has been
5 demonstrated between CT and LT immune subjects and CT
and LT mediated disease.

As shown in the examples below, mucosal immunity
may be achieved via the trancutaneous route. Mucosal
IgG and IgA can be detected in mice immunized with CT
10 transcutaneously. This may be important for
protection in diseases where the pathology occurs at
mucosal sites such as in LT or CT mediated disease,
where entry of the pathogenic organism occurs at a
mucosal site, or where mucosal infection is important
15 to pathogenesis.

It would be expected that transcutaneous
immunization against diseases such as influenza could
be effective either by inducing mucosal immunity or by
systemic immunity, or by a combination of immunity
20 such as humoral, cellular or mucosal.

Vaccines may be effective against host effects
such as the binding of erythrocytes to vascular
endothelium in malaria by inducing anti-sequestrin
antibodies.

25 Protective antibodies such as anti-hepatitis A, B
or hepatitis E antibodies may be induced by the
transcutaneous route using whole inactivated virus,
virus-derived subunits or recombinant products.

Protection against tetanus, diphtheria and other
30 toxin mediated diseases may be conferred by
transcutaneously induced anti-toxin antibodies. A
tetanus "booster" patch could be envisioned that
contained adjuvant such as CT and toxoids such as
tetanus and diphtheria, or fragments such as the
35 tetanus C fragment. Boosting could be achieved
following primary immunization by injection or
transcutaneous immunization with the same or similar

35

antigens. For injectable immunizations that induce
immunity but have potential side effects upon
boosting, a transcutaneous boost may be preferable.
Oral or nasal immunization may conceivably be boosted
5   using the transcutaneous route. Simultaneous use of
injectable and transcutaneous immunizations could also
be used.

        Vaccination has also been used as a treatment for
cancer and autoimmune disease. For example,
10   vaccination with a tumor antigen (e.g., prostate
specific antigen) may induce an immune response in the
form of antibodies, CTLs and lymphocyte proliferation
which allows the body's immune system to recognize and
kill tumor cells. Targeting dendritic cells, of which
15   Langerhans cells are a specific subset, has been shown
to be an important strategy in cancer immunotherapy.
Tumor antigens useful for vaccination have been
described for melanoma (U.S. Pat. Nos. 5,102,663,
5,141,742, and 5,262,177), prostate carcinoma (U.S.
20   Pat. No. 5,538,866), and lymphoma (U.S. Pat. Nos.
4,816,249, 5,068,177, and 5,227,159). Vaccination
with T-cell receptor oligopeptide may induce an immune
response that halts progression of autoimmune disease
(U.S. Pat. Nos. 5,612,035 and 5,614,192; Antel et al,
25   1996; Vandenbark et al, 1996). U.S. Pat. No.
5,552,300 also describes antigens suitable for
treating autoimmune disease.

        The following is meant to be illustrative of the
present invention; however, the practice of the
30   invention is not limited or restricted in any way by
the examples.

                          EXAMPLES

Immunization Procedure
35   BALB/c mice of 6 to 8 weeks were shaved with a
#40 clipper. This shaving could be done without any
signs of trauma to the skin. The shaving was done

36

from the mid-thorax to just below the nape of the neck. The mice were then allowed to rest for 24 hours. Prior to this the mice had been ear-tagged for identification, and pre-bled to obtain a sample of
5   pre-immune serum. Mice were also transcutaneously immunized without shaving by applying up to 50 µl of immunizing solution to each ear.

The mice were then immunized in the following way. Mice were anesthetized with 0.03-0.06 ml of a 20
10 mg/ml solution of xylazine and 0.5 ml of 100 mg/ml ketamine; mice were immobilized by this dose of anesthesia for approximately one hour. The mice were placed ventral side down on a warming blanket.

The immunizing solution was then placed on the
15 dorsal shaved skin of a mouse in the following manner: a 1.2 cm x 1.6 cm stencil made of polystyrene was laid gently on the back and a saline-wetted sterile gauze was used to partially wet the skin (this allowed even application of the immunizing solution), the
20 immunizing solution was then applied with a pipet to the area circumscribed by the stencil to yield a 2 cm^2 patch of immunizing solution. Alternatively, a fixed volume of immunizing solution was evenly applied to the shaved area or the ear. Care was used not to
25 scrape or rub the skin with the pipet tip. The immunizing solution was spread around the area to be covered with the smooth side of the pipet tip.

The immunizing solution (between about 100 µl to about 200 µl) was left on the back of the mouse for 60
30 to 180 minutes. At the end of 60 minutes, the mouse was held gently by the nape of the neck and the tail under a copious stream of lukewarm tap water, and washed for 10 seconds. The mouse was then gently patted dry with a piece of sterile gauze and a second
35 washing was performed for 10 seconds; the mouse was then patted dry a second time and left in the cage.

37

The mice appeared to exhibit no adverse effects from
the anesthesia, immunization, washing procedure, or
toxicity from the exotoxins. No skin irritation,
swelling or redness was seen after the immunization
5   and the mice appeared to thrive. Immunization using
the ear was performed as described above except that
fur was not removed prior to immunization.
Antigen
The following antigens were used for immunization
10   and ELISA, and were mixed using sterile PBS or normal
saline. Cholera toxin or CT (List Biologicals, Cat
#101B, lot #10149CB), CT B subunit (List Biologicals,
Cat #BT01, lot #CVXG-14E), CT A subunit (List
Biologicals, Cat #102A, lot #CVXA-17B), CT A subunit
15   (Calbiochem, Cat #608562); pertussis toxin, salt-free
(List Biologicals, lot #181120a); tetanus toxoid (List
Biologicals, lots #1913a and #1915a); *Pseudomonas*
exotoxin A (List Biologicals, lot #ETA25a); diphtheria
toxoid (List Biologicals, lot #15151); heat-labile
20   enterotoxin from *E. coli* (Sigma, lot #9640625); bovine
serum albumin or BSA (Sigma, Cat #3A-4503, lot #31F-
0116); and *Hemophilus influenza* B conjugate
(Connaught, lot#6J81401).

ELISA - IgG(H+L)
25   Antibodies specific for CT, LT, ETA, pertussis
toxin, diphtheria toxoid, tetanus toxoid, *Hemophilus
influenza* B conjugate, influenza, sequestrin, and BSA
were determined using ELISA in a technique similar to
Glenn et al (1995). All antigens were dissolved in
30   sterile saline at a concentration of 2 μg/ml. Fifty
microlilters of this solution (0.1 μg) per well was
put on IMMULON-2 polystyrene plates (Dynatech
Laboratories, Chantilly, VA) and incubated at room
temperature overnight. The plates were then blocked
35   with a 0.5% casein/0.05% Tween 20 blocking buffer
solution for one hour. Sera was diluted with 0.5%

38

casein/0.05% Tween 20 diluent; dilution series were
done in columns on the plate. Incubation was for 2
hours at room temperature.

    The plates were then washed in a PBS-0.05% Tween
5 20 wash solution four times, and goat anti-mouse
IgG(H+L) horseradish peroxidase (HRP)-linked (Bio-Rad
Laboratories, Richmond, CA, Cat #170-6516) secondary
antibody was diluted in casein diluent at a dilution
of 1/500 and left on the plates for one hour at room
10 temperature. The plates were then washed four times
in the PBS-Tween wash solution. One hundred
microliters of 2,2'-azino-di(3-ethyl-benzthiazolone)
sulphonic acid substrate (Kirkegaard and Perry) were
added to each well and the plates were read at 405 nm
15 after 20-40 minutes of development. Results are
reported as the geometric mean of individual sera and
standard error of the mean of ELISA units (the serum
dilution at which the absorbance in equal to 1.0) or
as individual antibody responses in ELISA units.

20 ELISA - IgG(γ), IgM(μ) and IgA(α)

    IgG(γ), IgM(μ) and IgA(α) anti-CT antibody levels
were determined using ELISA with a technique similar
to Glenn et al (1995). CT was dissolved in sterile
saline at a concentration of 2 μg/ml. Fifty
25 microliters of this solution (0.1 μg) per well were
put on IMMULON-2 polystyrene plates (Dynatech
Laboratories, Chantilly, VA) and incubated at room
temperature overnight. The plates were then blocked
with a 0.5% casein-Tween 20 blocking buffer solution
30 for one hour. Sera was diluted and casein diluent and
serial dilutions were done on the plate. This was
incubated for two hours at room temperature.

    The plates were then washed in a PBS-Tween wash
solution four times and goat anti-mouse IgG(γ) HRP-
35 linked (Bio-Rad Laboratories, Richmond, CA, Cat #172-

39

1038), goat anti-mouse IgM(μ) HRP-linked (BioRad
Laboratories, Richmond, CA, Cat #172-1030), or goat
anti-mouse IgA HRP-linked (Sigma, St. Louis, MO, Cat
#1158985) secondary antibody was diluted in casein

5   diluent in a dilution of 1/1000 and left on the plates
for one hour at room temperature. The plates were
then washed four times in a PBS-Tween wash solution.
One hundred microliters of 2,2'-azino-di(3-ethyl
benzthiazolone) sulphonic acid substrate from

10   (Kirkegaard and Perry, Gaithersburg, MD) were added to
the wells and the plates were read at 405 nm. Results
are reported as the geometric mean of individual sera
and standard error of the mean of ELISA units (the
serum dilution at which the absorbance in equal to

15   1.0).

ELISA - IgG Subclass
      Antigen-specific IgG (IgG1, IgG2a, IgG2b, and
IgG3) subclass antibody against CT, LT, ETA, and BSA
was performed as described by Glenn et al (1995). The

20   solid phase ELISA was performed in IMMULON-2
polystyrene plates (Dynatech Laboratories, Chantilly,
VA). Wells were incubated with the respective
antigens in saline overnight (0.1 μg/50 μl) and
blocked with 0.5% casein-Tween 20. Individual mouse

25   sera diluted in 0.5% casein were serially diluted, and
incubated at room temperature for four hours.
Secondary antibody consisted of horseradish
peroxidase-conjugated goat anti-mouse isotype-specific
antibody (IgG1, IgG2a, IgG2b, IgG3, The Binding Site,

30   San Diego, CA). A standard curve for each subclass
was determined using mouse myeloma IgG1, IgG2a, IgG2b,
and IgG3 (The Binding Site, San Diego, CA). Standard
wells were coated with goat anti-mouse IgG(H+L) (Bio-
Rad Laboratories, Richmond, CA, Cat #172-1054) to

35   capture the myeloma IgG subclass standards which were
added in serial dilutions. The myeloma IgG subclass

40

was also detected using the peroxidase-conjugated goat
anti-mouse subclass-specific antibody. Both the test
sera and myeloma standards were detected using 2,2'-
azino-di(3-ethyl-benzthiazolone) sulphonic acid

5   (Kirkegaard and Perry, Gaithersburg, MD) as substrate.
Absorbances were read at 405 nm. Individual antigen
specific subclasses were quantitated using the values
from the linear titration curve computed against the
myeloma standard curve and reported as μg/ml.

10   ELISA - IgE
         Antigen-specific IgE antibody quantitation was
performed using a protocol from Pharmingen Technical
Protocols, page 541 of the Research Products Catalog,
1996-1997 (Pharmingen, San Diego, CA). Fifty

15   microliters of 2 μg/ml purified anti-mouse IgE capture
mAb (Pharmingen, Cat# 02111D) in 0.1 M NaHCO$_3$ (pH 8.2)
were added to IMMUNO plates(Nunc, Cat #12-565-136).
Plates were incubated overnight at room temperature,
washed three times with PBS-Tween 20, blocked with 3%

20   BSA in PBS for two hours, and washed three times with
PBS-Tween. Sera were diluted in 1% BSA in PBS, added
at dilutions of 1/100, and diluted serially down the
columns (e.g., 1/100, 1/200, et cetera). Purified
mouse IgE standards (Pharmingen, Cat # 0312D) were

25   added with a starting dilution of 0.25 μg/ml and
serially diluted down the columns. Plates were
incubated for two hours and washed five times with
PBS-Tween.
         Biotinylated anti-mouse IgE mAB (Pharmingen, Cat

30   #02122D) to 2 μg/ml in 1% BSA in PBS, incubated for 45
minutes and washed five times with PBS-Tween. Avidin-
peroxidase (Sigma A3151, 1:400 of 1 mg/ml solution)
was added for 30 min and plates were washed six times
with PBS-Tween. Both the test sera and IgE standards

35   were detected using 2,2'-azino-di(3-ethyl-
benzthiazolone) sulphonic acid (Kirkegaard and Perry,

41

Gaithersburg, MD) as substrate. Absorbances were read
at 405 nm. Individual antigen specific subclasses
were quantitated using the values from the linear
titration curve computed against the IgE standard
5   curve and reported as µg/ml.

Liposome Preparation
        Where liposomes were included in the formulation
for transcutaneous immunization, multilamellar
liposomes composed of dimyristoyl phosphatidyl
10 choline, dimyristoyl phosphatidyl glycerol,
cholesterol were prepared according to Alving et al
(1993). Dimyristoyl phosphatidylcholine, dimyristoyl
phosphatidylglycerol, and cholesterol were purchased
from Avanti Polar Lipids Inc. (Alabaster, AL). Stock
15 solutions of the lipids in chloroform were removed
from -20°C freezer where they were stored.
        The lipids were mixed in a molar ratio of
0.9:0.1:0.75 dimyristoyl phosphatidyl choline,
dimyristoyl phosphatidyl glycerol, and cholesterol in
20 a pear shaped flask. Using a rotary evaporator, the
solvent was removed at 37°C under negative pressure
for 10 minutes. The flask was further dried under low
vacuum for two hours in a dessicator to remove
residual solvent. The liposomes were swollen at 37 mM
25 phospholipid using sterile water, lyophilized and
stored at -20°C. These liposomes were mixed in their
lyophilized state with normal saline (pH 7.0) to
achieve a designated phospholipid concentration in the
saline. Alternatively, the dried lipids were swollen
30 to make liposomes with normal saline (pH 7.0) and were
not lyophilized.

Example 1
        BALB/c mice at 6 to 8 weeks of age were immunized
transcutaneously as described above for "Immunization
35 Procedure", in groups of five mice. The mice were

42

immunized using 100 μl of immunization solution which
was prepared as follows: liposomes prepared as
described above for "Liposome Preparation" were mixed
with saline to form the liposomes. The pre-formed

5   liposomes were then diluted in either saline (liposome
alone group) or with CT in saline to yield an
immunizing solution containing liposomes at 10-150 mM
phospholipid with 100 μg of CT per 100 μl of
immunizing solution. CT was mixed in saline to make

10 an immunizing solution containing 100 μg of CT per 100
μg of solution for the group receiving CT alone.
Solutions were vortexed for 10 seconds prior to
immunization.

        The mice were immunized transcutaneously at 0 and

15 3 weeks. Antibody levels were determined using ELISA
as described above for "ELISA IgG(H+L)" 3 weeks after
the boosting immunization, and compared against pre-
immune sera. As shown in Table 1, the level of anti-
CT antibodies induced by CT without liposomes was not

20 different from the level of anti-CT antibodies
generated using liposomes except in the mice where 150
mM liposomes were used. CT in saline alone was able
to immunize mice against CT to produce high antibody
titers.

25

43

Table 1. Anti-CT antibodies

Group	ELISA Units	SEM
CT alone	27,482	(16,635-48,051)
CT + 150 mM Liposomes	4,064	*(2,845-5,072)
CT + 100 mM Liposomes	35,055	(25,932-44,269)
CT + 50 mM Liposomes	9,168	(4,283-12,395)
CT + 25 mM Liposomes	18,855	(12,294-40,374)
CT + 10 mM Liposomes	28,660	(18,208-31,498)
50 mM Liposomes	0	

* Significantly different from the Group CT alone (P<0.05)

5 Example 2

BALB/c mice at 6 to 8 weeks of age were immunized transcutaneously as described above for "Immunization Procedure", in groups of five mice. The mice were immunized at 0 and 3 weeks using 100 µl of

10 immunization solution prepared as follows: BSA was mixed in saline to make an immunizing solution containing 200 µg of BSA per 100 µl of saline for the group receiving BSA alone; BSA and CT were mixed in saline to make an immunizing solution containing 200

15 µg of BSA and 100 µg of CT per 100 µl of saline for the group receiving BSA and CT. Where liposomes were used, the liposomes were prepared as described above for "Liposome Preparation", and were first mixed with saline to form the liposomes. They were then diluted

20 in BSA or BSA and CT in saline to yield an immunizing solution containing liposomes at 50 mM phospholipid with 200 µg of BSA per 100 µl of immunizing solution, or 200 µg BSA + 100 µg CT per 100 µl of immunizing solution. Solutions were vortexed for 10 seconds

25 prior to immunization.

The antibodies were determined using ELISA as described above for "ELISA IgG(H+L)" on sera 3 weeks after the second immunization. The results are shown

44

in Table 2. BSA alone, with or without liposomes, was
not able to elicit an antibody response. However, the
addition of CT stimulated an immune response to BSA.
CT acted as a adjuvant for the immune response to BSA,
and anti-BSA antibodies of high titer were produced.

Table 2. Anti-BSA antibodies

Group	ELISA Units	SEM
BSA in saline	0	
BSA + 50 mM Liposomes	0	
CT + BSA in saline	8,198	(5,533-11,932)
CT + BSA + 50 mM	3,244	(128-3,242)

Example 3

BALB/c mice at 6 to 8 weeks of age were immunized
transcutaneously as described above for "Immunization
Procedure", in groups of five mice. The mice were
immunized at 0 and 3 weeks using 100 µl of
immunization solution prepared as follows: LT was
mixed in saline to make an immunizing solution
containing 100 µg of LT per 100 µl of saline for the
group receiving LT alone. Where liposomes were used
the liposomes prepared as described above for
"Liposome Preparation", and were first mixed with
saline to form the liposomes. The pre-formed
liposomes were then diluted in LT in saline to yield
an immunizing solution containing liposomes at 50 mM
phospholipid with 100 µg of LT per 100 µl of
immunizing solution. Solutions were vortexed for 10
seconds prior to immunization.

The anti-LT antibodies were determined using
ELISA as described above for "ELISA IgG(H+L)" 3 weeks
after the second immunization. The results are shown
in Table 3. LT was clearly immunogenic both with and
without liposomes, and no significant difference

between the groups could be detected. LT and CT are
members of the family of bacterial ADP-ribosylating
exotoxins (bAREs). They are organized as A:B
proenzymes with the ADP-ribosyltransferase activity
contained in the A subunit and the target cell binding
a function of the B subunit. LT is 80% homologous
with CT at the amino acid level and has a similar non-
covalently bound subunit organization, stoichiometry
(A:B5), the same binding target, ganglioside GM1, and
is similar in size (MW ~80,000). The similarities of
LT and CT appear to influence their immunogenicity by
the transcutaneous route as reflected by the similar
magnitude of the antibody response to both CT and LT
(Tables 1 and 3).

Table 3. Anti-LT antibodies

Group	ELISA Units	SEM
LT in saline	23,461	(20,262-27,167)
LT + 50 mM Liposomes	27,247	(19,430-38,211)

Example 4

C57Bl/6 mice at 6 to 8 weeks of age were
immunized transcutaneously as described above for
"Immunization Procedure", in groups of five mice. The
mice were immunized once using 100 µl of immunization
solution prepared as follows: LT was mixed in saline
to make an immunizing solution containing 100 µg of LT
per 100 µl of saline. The solution was vortexed for
10 seconds prior to immunization.

The anti-LT antibodies were determined using
ELISA as described above for "ELISA IgG (H+L)" 3 weeks
after the single immunization. The results are shown
in Table 4. LT was clearly immunogenic with a single
immunization and antibodies were produced by 3 weeks.
Rapid enhancement of antibody titers and responses to
single immunization would be a useful aspect of the

46

transcutaneous immunization method. It is conceivable
that a rapid single immunization would be useful in
epidemics, for travelers, and where access to medical
care is poor.

5   Table 4. Anti-LT antibodies

Mouse Number	ELISA Units
5141	6,582
5142	198
5143	229
5144	6,115
5145	17,542
Geo Mean	2,000

Example 5
       C57Bl/6 mice at 8 to 12 weeks of age were
immunized transcutaneously as described above for
10 "Immunization Procedure", in groups of five mice. The
mice were immunized once using 100 µl of immunization
solution prepared as follows: CT was mixed in saline
to make an immunizing solution containing 100 µg of CT
per 100 µl of saline. The solution was vortexed for
15 10 seconds prior to immunization.
       The anti-CT antibodies were determined using
ELISA as described above for "ELISA IgG (H+L)" 3 weeks
after the single immunization. The results are shown
in Table 5. CT was highly immunogenic with a single
20 immunization. Rapid enhancement of antibody titers
and responses to single immunization may be a useful
aspect of the transcutaneous immunization method. It
is conceivable that a rapid single immunization would
be useful in epidemics, for travelers, and where
25 access to medical care is poor.

47

Table 5. Anti-CT antibodies

Mouse Number	ELISA Units
2932	18,310
2933	30,878
2934	48,691
2935	7,824
Geo Mean	21,543

Example 6

5
BALB/c mice at 6 to 8 weeks of age were immunized transcutaneously as described above for "Immunization Procedure", in groups of five mice. The mice were immunized at 0 and 3 weeks using 100 μl of immunization solution prepared as follows: ETA was mixed in saline to make an immunizing solution

10
containing 100 μg of ETA per 100 μl of saline for the group receiving ETA alone. Where liposomes were used, the liposomes were prepared as described above for "Liposome Preparation", and were first mixed with saline to form the liposomes. The pre-formed

15
liposomes were then diluted with ETA in saline to yield an immunizing solution containing liposomes at 50 mM phospholipid with 100 μg of ETA per 100 μl of immunizing solution. Solutions were vortexed for 10 seconds prior to immunization.

20
The antibodies were determined using ELISA as described above for "ELISA IgG(H+L)" on sera 3 weeks after the second immunization. The results are shown in Table 6. ETA was clearly immunogenic both with and without liposomes, and no significant difference

25
between the groups could be detected. ETA differs from CT and LT in that ETA is a single 613 amino acid peptide with A and B domains on the same peptide and binds to an entirely different receptor, the α2-macroglobulin receptor/low density lipoprotein

48

receptor-related protein (Kounnas et al, 1992).
Despite the dissimilarities between ETA and CT in
size, structure, and binding target, ETA also induced
a transcutaneous antibody response.

5   Table 6. Anti-ETA antibodies

Group	ELISA Units	SEM
ETA in saline	3,756	(1,926-7,326)
ETA + 50 mM Liposomes	857	(588-1,251)

Example 7
        BALB/c mice at 6 to 8 weeks of age were immunized
transcutaneously as described above for "Immunization
10   Procedure", in groups of five mice. The mice were
immunized using 100 μl of immunization solution which
was prepared as follows: CT was mixed in saline to
make 100 μg of CT per 100 μl of immunizing solution,
LT was mixed in saline to make 100 μg of LT per 100 μl
15   of immunizing solution, ETA was mixed in saline to
make 100 μg of ETA per 100 μl of immunizing solution,
and CT and BSA were mixed in saline to make 100 μg of
CT per 100 μl of immunizing solution and 200 μg of BSA
per 100 μl of immunizing solution. Solutions were
20   vortexed for 10 seconds prior to immunization.
        The mice were immunized transcutaneously at 0 and
3 weeks and the antibody levels were determined using
ELISA as described above for "ELISA IgG Subclass",
three weeks after the boosting immunization and
25   compared against the pre-immune sera. The IgG
subclass response to CT, BSA and LT had similar levels
of IgG1 and IgG2a reflecting activation of T help from
both Th1 and Th2 lymphocytes (Seder and Paul, 1994),
whereas the IgG subclass response to ETA consisted of
30   almost exclusively IgG1 and IgG3, consistent with a
Th2-like response (Table 7). Thus, it appears that

49

all IgG subclasses can be produced using
transcutaneous immunization.

Table 7. IgG subclasses of induced antibodies

Imm. Antigen	Antibody Specificity	IgG1 (µg/µl)	IgG2a (µg/µl)	IgG2b (µg/µl)	IgG3 (µg/µl)
CT	CT	134	25	27	0
CT+BSA	BSA	108	17	12	5
LT	LT	155	28	10	8
ETA	ETA	50	0	1	10

5   Example 8
        BALB/c mice at 6 to 8 weeks of age were immunized
transcutaneously as described above for "Immunization
Procedure", in groups of five mice. The mice were
immunized using 100 µl of immunization solution which
10   was prepared as follows: LT was mixed in saline to
make an immunizing solution containing 100 µg of LT
per 100 µl of saline for the group receiving LT alone,
CT was mixed in saline to make an immunizing solution
containing 100 µg of CT per 100 µl of saline for the
15   group receiving CT alone, ETA was mixed in saline to
make an immunizing solution containing 100 µg of ETA
per 100 µl of saline for the group receiving ETA
alone, and BSA and CT were mixed in saline to make an
immunizing solution containing 100 µg of BSA and 100
20   µg of CT per 100 µl of saline for the group receiving
BSA and CT.
        The mice were immunized transcutaneously at 0 and
3 weeks and the antibody levels were determined using
ELISA as described above for "ELISA IgE", one week
25   after the boosting immunization and compared against
the pre-immune sera. As shown in Table 8, no IgE
antibodies were found although the sensitivity of
detection was 0.003 µg/ml. IgG antibodies were

50

determined in the same mice using "ELISA IgG(H+L)" on
sera 3 weeks after the second immunization. The IgG
antibody response to LT, ETA, CT and BSA are shown to
indicate that the animals were successfully immunized
5   and responded with high titers of antibodies to the
respective antigens.

Table 8. IgE antibodies to LT, ETA, CT and BSA

Group	Antibody Specificity	IgE (µg/ml)	IgG (ELISA Units)
LT	Anti-LT	0	23,461
ETA	Anti-ETA	0	3,756
CT	Anti-CT	0	39,828
CT + BSA	Anti-BSA	0	8,198

Example 9
10   BALB/c mice at 6 to 8 weeks of age immunized
transcutaneously as described above for "Immunization
Procedure", in groups of five mice. The mice were
immunized at 0 and 3 weeks using 100 ml of
immunization solution which was prepared as follows:
15 CT was mixed in saline to make an immunizing solution
containing 100 mg of CT per 100 ml of immunizing
solution. The immunization solution was vortexed for
10 seconds prior to immunization.
        The mice were immunized transcutaneously at 0 and
20 3 weeks and the antibody levels were determined using
ELISA as described above for "ELISA IgG(H+L)" and
"ELISA IgG(γ)". Determinations were done at 1 and 4
weeks after the initial immunization, and compared
against the pre-immune sera. As shown in Table 9,
25 high levels of anti-CT IgG(γ) antibodies were induced
by CT in saline. Small amounts of IgM could be
detected by using IgM(µ) specific secondary antibody.
By 4 weeks, the antibody response was primarily IgG.

Data are reported in ELISA units.

Table 9.   IgG(γ) and IgM(μ)

Imm. Group	Week	IgG(γ)	IgM(μ)
CT	1	72	168
CT	4	21,336	38
L()+CT	1	33	38
L()+CT	4	22,239	70

Example 10

BALB/c mice at 6 to 8 weeks of age were immunized transcutaneously as described above for "Immunization Procedure", in groups of five mice. The mice were immunized once using 100 µl of immunization solution prepared as follows: CT was mixed in saline to make an immunizing solution containing 100 µg of CT per 100 µl of saline. The solution was vortexed for 10 seconds prior to immunization. The mice were immunized transcutaneously at 0 and 3 weeks. Antibody levels were determined using ELISA as described above for "ELISA IgG (H+L)" 5 weeks after the boosting immunization, and compared against pre-immune sera. As shown in Table 10, serum anti-CT IgA antibodies were detected.

52

Table 10. Anti-CT IgA antibodies

Mouse Number	IgA (ng/ml)
1501	232
1502	22
1503	41
1504	16
1505	17

Example 11

     BALB/c mice at 6 to 8 weeks of age were immunized
5   transcutaneously as described above for "Immunization
Procedure", in groups of five mice. The mice were
immunized using 100 µl of immunization solution which
was prepared as follows: CT was mixed in saline to
make an immunizing solution containing 100 µg of CT
10 per 100 µl of immunizing solution. The immunization
solution was vortexed for 10 seconds prior to
immunization.

     The mice were immunized with 100 µl of immunizing
solution transcutaneously at 0 and 3 weeks and the
15 antibody levels were determined using ELISA as
described above for "ELISA IgG(H+L)" and "ELISA
IgG(γ)". Antibody determinations were done at 8 weeks
after the initial immunization and compared against
the pre-immune sera. As shown in Table 11, high
20 levels of serum anti-CT antibodies were induced by CT
in saline. Lung wash IgG could be detected by ELISA
using IgG(H+L) or IgG(γ) specific antibody. The
antibody found on the lung mucosal surface is diluted
by the lavage method used to collect mucosal antibody
25 and, thus, the exact amounts of antibody detected are
not as significant as the mere presence of detectable
antibody.

53

Lung washes were obtained after sacrificing the
mouse. The trachea and lungs were exposed by gentle
dissection and trachea was transected above the
bifurcation. A 22 gauge polypropylene tube was
5   inserted and tied off on the trachea to form a tight
seal at the edges. Half a milliliter of PBS was
infused using a 1 ml syringe attached to the tubing
and the lungs were gently inflated with the fluid.
The fluid was withdrawn and reinfused for a total of 3
10   rounds of lavage. The lung wash was then frozen at -
20°C.

Table 11 shows the IgG(H+L) and IgG(γ) antibody
response to cholera toxin in the sera and lung washes
at 8 weeks. Data are expressed in ELISA units.
15   Antibodies were clearly detectable for all mice in the
lung washes. The presence of antibodies in the mucosa
may be important for protection against mucosally
active diseases.

54

Table 11. Mucosal Antibody to CT

Animal#	Imm. Group	IgG(H+L)	IgG(γ)	Source
1501	CT	133	34	Lungs
1502	CT	75	12	Lungs
1503	CT	162	28	Lungs
1504	CT	144	18	Lungs
1505	CT	392	56	Lungs
	Geo Mean	156	26	
1501	CT	34,131	13,760	Sera
1502	CT	11,131	2,928	Sera
1503	CT	21,898	10,301	Sera
1504	CT	22,025	8,876	Sera
1505	CT	34,284	10,966	Sera
	Geo Mean	23,128	8,270	

Example 12

5   BALB/c mice were immunized transcutaneously at 0
and 3 weeks as described above for "Immunization
Procedure", in groups of four mice. Liposomes were
prepared as described above for "Liposome
Preparation", and were first mixed with saline to form
10   the liposomes. The pre-formed liposomes were then
diluted with either CT, CTA or CTB in saline to yield
an immunizing solution containing liposomes at 50 mM
phospholipid with 50 µg of antigen (CT, CTA or CTB)
per 100 µl of immunizing solution. Solutions were
15   vortexed for 10 seconds prior to immunization.
       The antibodies were determined using ELISA as
described above for "ELISA IgG(H+L)", one week after
the boosting immunization and compared against the
pre-immune sera. The results are shown in Table 12.
20   CT and CTB were clearly immunogenic whereas CTA was
not. Thus, the B subunit of CT is necessary and
sufficient to induce a strong antibody response.

55

Table 12. Antibodies to CT, CTA and CTB

Group	Anti-CT	Anti-CTA	Anti-CTB
CT + 50 mM Liposomes	12,636	136	7,480
CTB + 50 mM Liposomes	757	20	1,986
CTA + 50 mM Liposomes	0	0	0

Example 13

5   BALB/c mice were immunized transcutaneously as described above for "Immunization Procedure", in groups of five mice. Mice were immunized at 0 and 3 weeks with 100 µg of diphtheria toxoid and 10 µg of pertussis toxin per 100 µl of saline solution.

10   Solutions were vortexed for 10 seconds prior to immunization.

      The antibodies were quantitated using ELISA as described for "ELISA IgG(H+L)". Anti-diphtheria toxoid antibodies were detected only in animals

15   immunized with both pertussis toxin and diphtheria toxoid. The highest responder had anti-diphtheria toxoid antibody ELISA units of 1,038. Thus, a small amount of pertussis toxin acts as an adjuvant for diphtheria toxoid antigen. The toxoid alone did not

20   induce an immune response suggesting that the toxoiding process has affected the portion of the molecule responsible for the adjuvant effects found in the ADP-ribosylating exotoxin.

56

Table 13. Antibody to Diphtheria

Mouse Number	Immunizing Antigen	IgG ELISA Units
4731	DT + PT	1,039
4732	DT + PT	1
4733	DT + PT	28
4734	DT + PT	15
4735	DT + PT	20
4621	DT	0
4622	DT	0
4623	DT	0
4624	DT	0
4625	DT	0

Example 14

BALB/c mice were immunized transcutaneously as described above for "Immunization Procedure", in groups of five mice. Mice were immunized once at 0, 8 and 20 weeks with 50 µg of pertussis toxin (List, catalog # 181, lot #181-20a) per 100 µl of saline solution.

The antibodies were quantitated using ELISA as described for "ELISA IgG(H+L)". Anti-pertussis toxin antibodies were detected one week after the last boost in animals immunized with pertussis. All five animals had elevated levels of anti-petussis toxin antibody after the last immunization. Thus, pertussis toxin acts as an adjuvant for itself and induces PT-specific PT-specific IgG antibodies. The adjuvant effect of PT may be useful in combination vaccines such as Diphtheria/Pertussis/Tetanus/Hib in enhancing the antibody response to coadministered antigens as well as to PT itself.

Table 14. Antibody response to Pertussis toxin

Mouse Number	Antigen	2 weeks	21 weeks
5156	PT	14	256
5157	PT	22	330
5158	PT	17	303
5159	PT	33	237
5160	PT	75	418

Example 15

BALB/c mice were immunized transcutaneously as described above for "Immunization Procedure", in groups of five mice. Mice were immunized once at 0 weeks with 50 µg of tetanus toxoid and 100 µg of cholera toxin per 100 µl of saline solution.

The antibodies were quantitated using ELISA as described for "ELISA IgG(H+L)". Anti-tetanus toxoid antibodies were detected at 8 weeks in animal 5173 at 443 ELISA units.

Example 16

The possibility that oral immunization occurred through grooming after epicutaneous application and subsequent washing of the site of application was evaluated using ^{125}I-labeled CT to track the fate of the antigen/adjuvant. Mice were anesthetized, transcutaneously immunized as described above for "Immunization Procedure" with 100 µg of ^{125}I-labeled CT (150,000 cpm/µg CT). Control mice remained anesthetized for 6 hours to exclude grooming, and experimental mice were anesthetized for one hour and then allowed to groom after washing. Mice were sacrificed at 6 hours and organs weighed and counted for ^{125}I on a Packard gamma counter. A total of 2-3 µg of CT was detected on the shaved skin at the site of immunization (14,600 cpm/µg tissue) while a maximum of

58

0.5 μg of CT was detected in the stomach (661 cpm/μg

tissue) and intestine (9 cpm/μg tissue).

Oral immunization (n=5) with 10 μg if CT in

saline at 0 and 3 weeks (without NaHCO₃) induced a 6

5   week mean IgG antibody response of < 1,000 ELISA units

whereas transcutaneous immunization with 100 μg of CT,

shown above to result in less than 5 μg of CT retained

in the skin after washing, resulted in an anti-CT

response of 42,178 ELISA units at 6 weeks. Induction

10   of an immune response to orally fed CT requires the

addition of NaHCO₃ to the immunizing solution (Piece,

1978; Lycke and Holmgren, 1986). Thus, oral

immunization does not significantly contribute to the

antibodies detected when CT is applied epicutaneously

15   to the skin.

Example 17

In vivo evidence of Langerhans cell activation

was obtained using cholera toxin (CT) in saline

applied epicutaneously to the skin, specifically the

20   ears of the mouse, where large populations of

Langerhans cells can be readily visualized (Enk et al,

1993; Bacci et al, 1997), and staining for major

histocompatibility complex (MHC) class II molecules

which is upregulated in activated Langerhans cells

25   (Shimada et al, 1987).

BALB/c mouse ears were coated on the dorsal side

with either 100 μg of CT in saline, 100 μg of CTB in

saline, saline alone, or an intradermal injection of

the positive controls 100 pg LPS or 10 μg TNFα, for

30   one hour while the mouse was anesthetized. The ears

were then thoroughly washed and, after 24 hours, the

ears were removed and epidermal sheets were harvested

and stained for MHC class II expression as described

by Caughman et al (1986). Epidermal sheets were

35   stained with MKD6 (anti-I-A^d) or negative control Y3P

59

(anti-I-A^k), and goat anti-mouse FITC F(ab)$_2$ was used as a second step reagent. Mice transcutaneously immunized on the ear (as described above without shaving) had previously been found to have anti-CT

5   antibodies of 7,000 ELISA units three weeks after a single immunization.

Enhanced expression of MHC class II molecules as detected by staining intensity, the reduced number of Langerhans cells (especially with cholera toxin), and

10   changes in Langerhans cell morphology were found in the epidermal sheets of the mice immunized with CT and CTB comparable to controls (Fig. 1), suggesting that the Langerhans cells were activated by the epicutaneously applied cholera toxin (Aiba and Katz,

15   1990; Enk et al, 1993).

Example 18

Langerhans cells represent the epidermal contingent of a family of potent accessory cells termed 'dendritic cells'. Langerhans cells (and

20   perhaps related cells in the dermis) are thought to be required for immune responses directed against foreign antigens that are encountered in skin. The 'life cycle' of the Langerhans cell is characterized by at least two distinct stages. Langerhans cells in

25   epidermis (the 'sentinels') can ingest particulates and process antigens efficiently, but are weak stimulators of unprimed T cells. In contrast, Langerhans cells that have been induced to migrate to lymph nodes after contact with antigen in epidermis

30   (the 'messengers') are poorly phagocytic and have limited antigen-processing capabilities, but are potent stimulators of naive T cells. If Langerhans cells are to fulfill both their 'sentinel' and 'messenger' roles, they must be able to persist in

35   epidermis, and also be able to exit epidermis in a controlled fashion after exposure to antigen. Thus,

regulation of Langerhans cell-keratinocyte adhesion
represents a key control point in Langerhans cell
trafficking and function.

   Langerhans cells express E-cadherin (Blauvelt et
5 al, 1995), a homophilic adhesion molecule that is
prominently represented in epithelia. Keratinocytes
also express this adhesion molecule, and E-cadherin
clearly mediates adhesion of murine Langerhans cells
to keratinocytes in vitro. It is known that E-
10 cadherin is involved in the localization of Langerhans
cells in epidermis. See Stingl et al (1989) for a
review of the characterization and properties of
Langerhans cells and keratinocytes.

   The migration of epidermal Langerhans cells (LC)
15 and their transport of antigen from the skin to
draining lymph nodes are known to be important in the
induction of cutaneous immune responses, such as
contact sensitization. While in transit to the lymph
nodes, Langerhans cells are subject to a number of
20 phenotypic changes required for their movement from
the skin and acquisition of the capacity for antigen
presentation. In addition to the upregulation of MHC
class II molecules, are alterations in the expression
of adhesion molecules that regulate interactions with
25 the surrounding tissue matrix and with T lymphocytes.
The migration of the Langerhan cell is known to be
associated with a marked reduction in the expression
of E-cadherin (Schwarzenberger and Udey, 1996, and a
parallel upregulation of ICAM-1 (Udey, 1997).

30   Transcutaneous immunization with bacterial ADP
ribosylating exotoxins (bARE's) target the Langerhans
cells in the epidermis. The bAREs activate the
Langerhans cell, transforming it from its sentinel
role to its messenger role. Ingested antigen is then
35 taken to the lymph node where it is presented to B and
T cells (Streilein and Grammer, 1989; Kripke et al,
1990; Tew et al, 1997). In the process, the epidermal

61

Langerhans cell matures into an antigen-presenting
dendritic cell in the lymph node (Schuler and
Steinman, 1985); lymphocytes entering a lymph node
segregate into B-cell follicles and T-cell regions.

5   The activation of the Langerhans cell to become a
migratory Langerhans cell is known to be associated
with not only a marked increase in MHC class II
molecules, but also marked reduction in the expression
of E-cadherin, and upregulation of ICAM-1.

10   We envision that cholera toxin (CT) and its B
subunit (CTB) upregulate the expression of ICAM-1 and
downregulate the expression of E-cadherin on
Langerhans cells as well as upregulate the expression
of MHC class II molecules on the Langerhans cell. CT

15 or CTB acts as an adjuvant by freeing the sentinel
Langerhans cell to present antigens such as BSA or
diphtheria toxoid phagocytosed by the Langerhans cell
at the same location and time as the encounter with
the CT or CTB when they are acting as adjuvant. The

20 activation of a Langerhans cells to upregulate the
expression of ICAM-1 and dowregulate the expression of
E-cadherin may be mediated by cytokine release
including TNFα and IL-1β from the epidermal cells or
the Langerhans cells themselves.

25   This method of adjuvancy for transcutaneous
immunization is envisioned to work for any compound
that activates the Langerhans cell. Activation could
occur in such manner as to downregulate the E-cadherin
and upregulate ICAM-1. Langerhans cells would then

30 carry antigens made of mixtures of such Langerhans
cell-activating compounds and antigens (such as
diphtheria toxoid or BSA) to the lymph nodes where the
antigens are presented to T cells and evoke an immune
response. Thus, the activating substance such as a

35 bARE can be used as an adjuvant for an other wise
transcutaneously non-immunogenic antigen such as
Diphtheria toxoid by activating the Langerhans cell to

62

phagocytose the antigen such as diphtheria toxoid,
migrate to the lymph node, mature into a dendritic
cell, and present the antigen to T cells.

The T-cell helper response to antigens used in
5   transcutaneous immunization may be influenced by the
application of cytokines and/or chemokines. For
example, interleukin-10 (IL-10) may skew the antibody
response towards a Th2 IgG1/IgE response whereas anti-
IL-10 may enhance the production of IgG2a
10   (Bellinghausen et al, 1996).

Example 19
Sequestrin is a molecule expressed on the surface
of malaria-infected erythrocytes which functions to
15   anchor the malaria parasitized red blood cell to
vascular endothelium. This is essential for parasite
survival and contributes directly to the pathogenesis
of *P. falciparum* malaria in children dying of cerebral
malaria. In cerebral malaria, the brain capillaries
20   become plugged with vast numbers of parasitized red
blood cells due to the specific interaction of the
sequestrin molecule with the host endothelial receptor
CD36. Ockenhouse et al identified both the host
receptor CD36 and parasite molecule (sequestrin) which
25   mediates this receptor-ligand interaction. Ockenhouse
et al have cloned and expressed as *E. coli*-produced
recombinant protein the domain of the sequestrin
molecule which interacts with the CD36 receptor. A
truncated 79 amino acid sequestrin product was used in
30   the example below.
Active immunization with recombinant sequestrin
or DNA encoding the gene for sequestrin should elicit
antibodies which block the adhesion of malaria
parasitized erythrocytes to host endothelial CD36 and
35   thereby prevent completion of parasite life cycle
leading to parasite death due to its inability to bind
to endothelium. The strategy is to develop a method

63

of immunization which elicits high titer blocking antibodies. One such method is the deliver the vaccine transcutaneously. Measurement of both total antibody titers as well as blocking activity and
5   opsonization forms the basis for this approach with transcutaneous immunization. The recombinant sequestrin protein used in the present experiments is 79 amino acids long (~18 kDa) and comprises the CD36-binding domain of the molecule. We have also
10   constructed a naked DNA construct comprised of this domain and have elicited antibodies using epidermal gene gun delivery.

BALB/c mice (n=3) were immunized transcutaneously as described above for "Immunization Procedure". The
15   mice were immunized at 0 and 8 weeks using 120 μl of immunization solution prepared as follows: a plasmid encoded for *P. falciparum* sequestrin was mixed in saline to make an immunizing solution containing 80 μg of plasmid, 80 μg of CT (List Biologicals) per 100 μl
20   of saline. One hundred-twenty μl was applied to the untagged ear after gently cleansing the ear with an alcohol swab (Triad Alcohol pad, 70% isopropyl alcohol). The immunizing solution was not removed by washing.
25   The antibodies to sequestrin were determined using ELISA as described above for "ELISA IgG(H+L)" on sera collected from the tail vein at weeks 3, 4, 7 and 9 after the primary immunization. The results are shown in Table 15.
30   Sequestrin DNA with CT induced a detectable antibody response to the expressed protein after the second boosting immunization. For immunization to occur, the protein needs to be expressed and processed by the immune system. Thus, CT acted as an adjuvant
35   for the immune response to sequestrin protein expressed by the plasmid encoding for sequestrin.

64

DNA vaccines have been shown to elicit
neutralizing antibodies and CTLs in non-human primates
to diseases such as malaria (Gramzinski, Vaccine,
15:913-915, 1997) and HIV (Shriver et al, Vaccine
5   15:884-887, 1997) and have demonstrated protection to
varying degrees in several models (McClements et al,
Vaccine, 15:857-60, 1997). DNA immunization through
the skin could be expected to elicit responses similar
to that of the gene gun which targets the skin immune
10 system (Prayaga et al, Vaccine, 15:1349-1352, 1997).

Table 15.   Serum antibody against sequestrin (Seq) protein
in animals immunized with Seq DNA and Cholera toxin (CT)

Animal #	Imm. Group	IgG (H+L) ELISA Units			
		week 3	week 4	week 7	week 9
8966	Seq DNA/CT	58	80	33	–
8967	Seq DNA/CT	76	81	41	146
8968	Seq DNA/CT	54	33	26	–
	Geo Mean	62	60	33	
pooled prebleed	–	40			

Example 20
        BALB/c mice were immunized transcutaneously as
15 described above for "Immunization Procedure", in
groups of five mice, using sequestrin. The mice were
immunized at 0, 2 and 8 weeks using 100 µl of
immunization solution prepared as follows: at 0 weeks
the mice were immunized with 59 µg of CT and 192 µg of
20 sequestrin in 410 µl for the group receiving
sequestrin and CT, 192 µg in 410 µl for sequestrin
alone, and 120 µg of CTB and 250 µg of sequestrin in
520 µl for the group receiving sequestrin and CTB.
Two weeks later the mice were boosted with 345 µl of
25 saline containing either 163 µg sequestrin for the
sequestrin alone group, 345 µl of saline containing
163 µg sequestrin with 60 µg of CT for the CT plus
sequestrin group, 345 µl of saline containing 163 µg
sequestrin and 120 µg of CTB for the sequestrin plus

CTB group. In the second boost the mice were given
120 µg of sequestrin for the sequestrin alone group,
120 µg of sequestrin and 120 µg of CT for the CT plus
sequestrin group and 120 µg of sequestrin and 120 µg

5   of CTB for the sequestrin plus CTB group.

The antibodies were determined using ELISA as
described above for "ELISA IgG(H+L)" on sera 3, 5, 7,
9, 10, 11 and 15 weeks after the first immunization.
The results are shown in Table 16. Sequestrin alone

10   induced a small but detecable antibody response.
However, the addition of CT stimulated a far stronger
immune response to sequestrin and CTB induced an
immune response that was superior to sequestrin alone.
CT and CTB acted as adjuvants for the immune response

15   to sequestrin, a recombinant protein.

WO 98/20734 PCT/US97/21324

66

Table 16. Seq, Seq + Cholera toxin (CT), or Seq + Cholera toxin B (CTB)

Animal #	Immunization Group	Detecting Antigen	IgG (H+L) ELISA Units							
			prebleed	week 3	week 5	week 7	week 8	week 9	week 11	week 15
2861	Seq	Seq		7	7	20	32	709	431	408
2862	Seq	Seq		8	5	14	136	33	4	6
2863	Seq	Seq		28	63	38	393	467	348	459
2864	Seq	Seq		5	9	26	102	32	13	11
2865	Seq	Seq		9	19	76	111	100	53	98
	Geo Mean			9	13	29	114	129	54	65
2866	Seq/CT	Seq		923	1145	125	639	43679	28963	42981
2867	Seq/CT	Seq		73	84	154	ND	9428	20653	27403
2868	Seq/CT	Seq		805	370	1447	1105	ND	13169	7677
2869	Seq/CT	Seq		175	760	1317	768	113792	118989	270040
2870	Seq/CT	Seq		153	158	535	241	3245	ND	4277
	Geo Mean			271	336	456	601	19747	31115	25279
2871	Seq/CTB	Seq		8	3	87	40	22	29	192
2872	Seq/CTB	Seq		4	6	24	22	35	24	34
2873	Seq/CTB	Seq		107	138	128	51	2074	2283	2296
2874	Seq/CTB	Seq		6	7	22	18	41	40	457
2875	Seq/CTB	Seq		515	504	1910	1744	ND	7148	5563
	Geo Mean			25	25	102	68	91	214	520
pool										

5

Example 21

BALB/c mice were immunized transcutaneously as described above for "Immunization Procedure", in groups of five mice. The mice were immunized at 0 weeks using 100 µl of immunization solution prepared as follows: FLUSHIELD (Wyeth-Ayerst, purified subvirion, 1997-98 formula, lot #U0980-35-1) was lyophilized and was mixed in saline to make an immunizing solution containing 90 µg of FLUSHIELD subvirion per 100 µl of saline for the group receiving influenza alone; influenza and CT were mixed in saline to make an immunizing solution containing 90 µg of FLUSHIELD antigens and 100 µg of CT per 100 µl of saline for the group receiving influenza and CT.

The antibodies were determined using ELISA as described above for "ELISA IgG(H+L)" on sera 3 weeks after the first immunization. The results are shown in Table 17. Influenza alone did not induce an antibody response. However, the addition of CT stimulated a far stronger immune response which was superior to that observed influenza alone. Thus CT acted as an adjuvant for the immune response to FLUSHIELD, subvirion influenza vaccine, a mixture of virally derived antigens.

68

Table 17.   Serum antibody against Influenza (Inf)
       types A and B in animals immunized with
        Inf alone or Inf + Cholera toxin (CT)

Animal #	Imm. Group	IgG (H+L) ELISA Units week 3
8601	CT/Inf	144
8602	CT/Inf	14
8603	CT/Inf	1325
8604	CT/Inf	36
8605	CT/Inf	29
	Geo Mean	77
8606	Inf	17
8607	Inf	16
8608	Inf	20
8609	Inf	23
8610	Inf	23
	Geo Mean	20

Example 22

LT is in the family of ADP-ribosylating exotoxins
and is similar to CT in molecular weight, binds to
5   ganglioside GM1, is 80% homologous with CT and has a
similar A:B5 stoichiometry. Thus, LT was also used as
an adjuvant for DT in transcutaneous immunization.
BALB/c mice (n=5) were immunized as described above at
0, 8 and 18 weeks with a saline solution containing
10   100 μg of LT (Sigma, catalog #E-8015, lot 17hH12000
and 100 μg CT (List Biologicals, catalog #101b) in 100
μl of saline. LT induced a modest response to DT as
shown in Table 18.

ETA (List Biologicals, lot #ETA 25A) is in the
15   family of ADP-ribosylating exotoxins, but is a single
polypeptide that binds to a different receptor. One
hundred μg of ETA was delivered in 100 μl of a saline
solution containing 100 μg of CT to BALB/c mice on the
back as previously described at 0, 8 and 18 weeks.
20   ETA boosted the response to DT at 20 weeks. Thus,
other ADP-ribosylating exotoxins were able to act as

69

adjuvants for coadministered proteins (Table 18).

Table 18.   Kinetics of Diphtheria toxoid (DT) antibody
                    titers in animals immunized with
          Pseudomonas aeruginosa exotoxin A (ETA) and DT or
            E. coli heat labile enterotoxin (LT) and DT

Animal #	Immunization Group	Detecting Antigen	IgG (H+L) ELISA Units	
			prebleed	week 20
5146	ETA/DT	DT		31718
5147	ETA/DT	DT		48815
5148	ETA/DT	DT		135
5149	ETA/DT	DT		34
5150	ETA/DT	DT		258
		Geo Mean		1129
5136	LT/DT	DT		519
5137	LT/DT	DT		539
5138	LT/DT	DT		38
5139	LT/DT	DT		531
5140	LT/DT	DT		901
		Geo Mean		348
pool			3	

Example 23

5   BALB/c mice were immunized transcutaneously as
described above for "Immunization Procedure" in groups
of five mice. Mice were immunized at 0 weeks, 8 weeks
and 18 weeks with 100 µl saline containing 100 µg
Cholera toxin (List Biologicals, catalog #101B, lot
10   #10149CB), 50 µg Tetanus toxoid (List Biologicals,
catalog # 191B, lots #1913a and 1915b) and 83 µg
Diphtheria toxoid (List Biologicals, catalog #151, lot
#15151).

       The antibodies against CT, DT, and TT were
15   quantitated using ELISA as described for "ELISA IgG
(H+L)". Anti-CT, DT, or TT antibodies were detected
at 23 weeks following the primary immunization. Anti-
Diphtheria toxoid and Cholera toxin antibodies were
elevated in all immunized mice. The highest responder
20   had anti-tetanus toxoid antibody ELISA units of 342,
approximately 80 times the level of antibody detected

70

in sera of unimmunized animals. Thus, a combination
of unrelated antigens (CT/TT/DT) can be used to
immunize against the individual antigens. This
demonstrates that Cholera toxin can be used as an
5   adjuvant for multivalent vaccines.

Table 19. Serum antibody in animals immunized simultaneously
with Cholera toxin, Tetanus toxoid, and Diphtheria toxoid

Animal #	Imm. Group	detecting antigen	IgG (H+L) ELISA Units	
			prebleed	23 weeks
5176	CT/TT/DT	CT		7636
5177	CT/TT/DT	CT		73105
5179	CT/TT/DT	CT		126259
5216	CT/TT/DT	CT		562251
5219	CT/TT/DT	CT		66266
pool			≤3	
	Geo Mean			76535
5176	CT/TT/DT	DT		64707
5177	CT/TT/DT	DT		17941
5179	CT/TT/DT	DT		114503
5216	CT/TT/DT	DT		290964
5219	CT/TT/DT	DT		125412
pool			≤4	
	Geo Mean			86528
5176	CC/TT/DT	TT		21
5177	CC/TT/DT	TT		30
5179	CT/TT/DT	TT		342
5216	CT/TT/DT	TT		36
5219	CT/TT/DT	TT		30
pool			≤2	
	Geo Mean			47

Example 25

Transcutaneous immunization using CT induces
10   potent immune responses. The immune response to an
intramuscular injection and oral immunization was
compared to transcutaneous immunization using CT as
adjuvant and antigen. Twenty-five μg of CT (List
Biologicals, catalog #101b) dissolved in saline was
15   administered orally in 25 μl to BALB/c mice (n=5)
using a 200 μl pipette tip. The mice readily
swallowed the immunization solution. Twenty-five μl
of 1 mg/ml CT in saline was administered on the ear as
described to the group labeled transcutaneous.

71

Twenty-five µg of CT in saline was injected IM into
the anterior thigh in the group labeled intramuscular.

The mice injected IM with CT developed marked
swelling and tenderness at the injection site and
5   developed high levels of anti-CT antibodies. Mice
immunized transcutaneously had no redness or swelling
at the site of immunization and developed high levels
of ant-CT antibodies. Mice immunized orally developed
far lower levels of antibodies compared to the mice
10   immunized transcutaneously. This indicates that oral
immunization through grooming in the transcutaneously
immunized mice does not account for the high levels of
antibodies induced by transcutaneous immunization.
Overall, the transcutaneous route of immunization is
15   superior to either oral or IM immunization as high
levels of antibodies are achieved without adverse
reactions to the immunization.

Table 20. Kinetics of Cholera toxin antibody titers in animals
immunized by the transcutaneous, oral, or intramuscular route

Animal #	Immunization Route	IgG (H+L) ELISA Units	
		prebleed	week 6
8962	transcutaneous		23489
8963	transcutaneous		30132
8964	transcutaneous		6918
8965	transcutaneous		20070
8825	transcutaneous		492045
pool		16	
	Geo Mean		34426
8951	oral		743
8952	oral		4549
8953	oral		11329
8954	oral		1672
pool		14	
	Geo Mean		2829
8955	intramuscular		35261
8958	intramuscular		607061
8959	intramuscular		452966
8850	intramuscular		468838
8777	intramuscular		171648
pool		12	
	Geo Mean		239029

72

Example 26

BALB/c mice were immunized transcutaneously as
described above for "Immunization Procedure", in
groups of five mice. The mice were immunized at 0, 8
5   and 20 weeks using 100 µl of immunization solution
prepared as follows: Hib conjugate (Connaught, lot
#6J81401, 86 µg/ml) was lyophilized in order to
concentrate the antigen. The lyophilized product was
mixed in saline to make an immunizing solution
10 containing 50 µg of Hib conjugate per 100 µl of saline
for the group receiving Hib conjugate alone; Hib
conjugate and CT were mixed in saline to make an
immunizing solution containing 50 µg of Hib conjugate
and 100 µg of CT per 100 µl of saline for the group
15 receiving Hib conjugate and CT.

The antibodies were determined using ELISA as
described above for "ELISA IgG(H+L)" on sera 3 weeks
after the second immunization. The results are shown
in Table 21. Hib conjugate alone induced a small but
20 detectable antibody response. However, the addition
of CT stimulated a far stronger immune response to Hib
conjugate. CT acted as an adjuvant for the immune
response to Hib conjugate. This indicates that a
polysaccharide conjugate antigen can be used as a
25 transcutaneous antigen by the method described.

Table 21. Antibody to Haemophilus influenzae b (Hib)

Animal #	Imm. Group	IgG (H+L) ELISA Units
5211	Hib	57
5212	Hib	29
5213	Hib	28
5214	Hib	63
5215	Hib	31
	Geo Mean	39
5201	CT/Hib	1962
5202	CT/Hib	3065
5203	CT/Hib	250
5204	CT/Hib	12
5205	CT/Hib	610
	Geo Mean	406
pool prebleed	–	1

Example 27

Emulsions, creams and gels may provide practical advantages for convenient spreading of the immunizing compound over the skin surface, over hair or body creases. Additionally such preparations may provide advantages such as occlusion or hydration which may enhance the efficiency of the immunization.

Heat labile entertoxin (LT) from *E. coli* (Sigma, catalog #E-8015, lot 17hH1200) was used to compare the efficiency of transcutaneous immunization using a simple saline solution and a commonly available petroleum base ointment, AQUAPHOR, which "can be used alone or in compounding virtually any ointment using aqueous solutions or in combination with other oil based substances and all common topical medications." (page 507 PDR, for Non-prescriptions Drugs, 1994, 15th Ed.). Mice were treated with a range of doses to evaluate the relative antibody response for the decreasing doses in the comparative vehicles.

BALB/c mice were immunized as described above.

74

except that the immunizing solution was applied for 3
hours on the back. Saline solutions of LT were
prepared to deliver a 50 µl dose of solution and
either 100 µg, 50 µg, 25 µg or 10 µg of antigen in the
5   solution, using a 2 mg/ml, 1 mg/ml, 0.5 mg/ml or 0.2
mg/ml solution, respectively. After 3 hours the back
was gently wiped using wetted gauze to remove the
immunizing solution.
        The water in oil preparation was performed as
10   follows: equal volumes of AQUAPHOR and antigen in
saline solution were mixed in 1 ml glass tuberculin
syringes with luer locks using a 15 gauge emulsifying
needle connecting the two syringes and mixing until
the mixture was homogenous. A 4 mg/ml, 2 mg/ml, 1
15   mg/ml or 0.5 mg/ml solution of LT in saline was used,
respectively, to mix with an equal volume of AQUAPHOR.
50 µl of this mixture was applied to the shaved back
for three hours and then gently removed by wiping with
gauze. Doses of antigen for the water in oil LT
20   containing emulsions were weighed in order to deliver
50 µl. The weight per volume ratio was calculated by
adding the specific gravity of saline (1.00 g/ml) and
AQUAPHOR, 0.867 gm/ml, and dividing the sum by 2 for a
final specific gravity of 0.9335 gm/ml. Approximately
25   47 mg of water in oil emulsion containing LT was
delivered to the mouse for immunization.
        A dose-response relationship was evident for both
saline and water in oil emulsion delivered LT (Table
22). One hundred µg induced the highest level of
30   antibodies and 10 µg induced a lower but potent immune
response. Water in oil emulsified LT induced a
similar response to LT in saline and appears to offer
a convenient delivery mechanism for transcutaneous
immunization. Similarly, gels, creams or more complex
35   formulations such as oil-in water-in-oil could be used
to deliver antigen for transcutaneous immunization.

Such compositions could be used in conjunction with patches, occlusive dressings, or reservoirs and may allow long-term application or short term application of the immunizing antigen and adjuvant.

Table 22. Serum antibody against *E. coli* heat-labile enterotoxin (LT) in animals immunized with varying doses of LT in a saline or AQUAPHOR emulsion

Imm Group	emulsion	animal id#	IgG (H+L) Elisa Units pre-bleed	week 3	emulsion	animal id#	IgG (H+L) Elisa Units pre-bleed	week 3
LT 100 µg	saline	8741		18434	aquaphor	8717		6487
LT 100 µg	saline	8742		16320	aquaphor	8719		4698
LT 100 µg	saline	8743		19580	aquaphor	8774		18843
LT 100 µg	saline	8744		19313	aquaphor	8775		18217
LT 100 µg	saline	8745		22875	aquaphor	8861		16230
LT 100 µg		pool	32			pool	54	
Geo Mean				19190				11117
	saline	8736		19129	aquaphor	8721		4160
LT 50 µg	saline	8737		3975	aquaphor	8722		12256
LT 50 µg	saline	8738		6502	aquaphor	8725		12262
LT 50 µg	saline	8739		6224	aquaphor	8771		12982
LT 50 µg	saline	8740		18449	aquaphor	8772		15246
LT 50 µg		pool	54			pool	57	
Geo Mean				8929				10435
	saline	8768		3274	aquaphor	8727		3585
	saline	8731		3622	aquaphor	8728		3
LT 25 µg	saline	8732		557	aquaphor	8729		4206
LT 25 µg	saline	8733		626	aquaphor	8862		7353
LT 25 µg	saline	8734		1725	aquaphor	8769		5148
LT 25 µg		pool	56			pool	53	
LT 25 µg				1481				1114
Geo Mean	saline	8848		621	aquaphor	8748		1968
	saline	8849		475	aquaphor	8749		1935
	saline	8757		858	aquaphor	8750		646
LT 10 µg	saline	8759		552	aquaphor	8747		1569
LT 10 µg	saline	8760		489	aquaphor	8764		1
LT 10 µg		pool	43			pool	39	
LT 10 µg				585				329
LT 10 µg								
Geo Mean								

Example 28

Mice were immunized transcutaneously as described above for "Immunization Procedure", in groups of five mice. Mice were immunized at 0, 8 and 18 weeks with 100 µl saline containing 50 µg Tetanus toxoid (List Biologicals, catalog #191B, lots #1913a and #1915b) and 83 µg Diphtheria toxoid (List Biologicals, catalog #151, lot #15151) alone or in combination with 100 µg

76

Cholera toxin (List Biologicals, catalog #101B, lot #10149CB).

　　　Anti-Diphtheria toxoid antibodies were quantitated using ELISA as described for "ELISA IgG (H+L)". Elevated levels of anti-toxoid antibodies were detected in animals given immunized with either TT/DT or CT/TT/DT. However, the antibody titers were far superior in animals in which CT was included as an adjuvant. This anti-Toxoid titer was obviously increased in both groups after each subsequent immunization (8 and 18 weeks). Thus while DT can induce a small but significant response against itself the magnitude of the response can be increased by 1) inclusion of cholera toxin as an adjuvant and 2) boosting with the adjuvant Cholera toxin and antigen (Diphtheria toxoid). Classic boosting responses are dependent on T-cell memory and the boosting of the anti-DT antibodies in this experiment indicate that T-cells are engaged by transcutaneous immunization.

Table 23. Kinetics of Diphtheria toxoid (DT) antibody titers in animals immunized with Tetanus toxoid (TT) and DT or Cholera toxin (CT), TT, and DT

Animal #	Immunization Group	Detecting Antigen	prebleed	week 2	week 4	week 6	week 8	week 10	week 14	week 17	week 18	week 20	week 23
								IgG (H+L) ELISA Units					
5196	TT/DT	DT		7	12	18	23	49	56	33	18	219	166
5197	TT/DT	DT		5	11	11	10	15	17	16	17	125	75
5198	TT/DT	DT		13	20	16	-	28	25	27	7	48	172
5199	TT/DT	DT		13	8	10	10	11	22	12	217	178	263
5200	TT/DT	DT		4	10	4	7	120	149	127	-	17309	14537
	Geo Mean			7	12	10	11	31	38	29	26	332	382
5176	CT/TT/DT	DT		8	26	21	14	3416	5892	1930	1826	63087	64704
5177	CT/TT/DT	DT		8	6	7	8	424	189	149	175	16416	17941
5179	CT/TT/DT	DT		8	3	4	8	4349	1984	2236	1921	124239	114503
5216	CT/TT/DT	DT		12	5	9	11	3238	2896	2596	1514	278281	290964
5219	CT/TT/DT	DT		8	15	13	12	5626	4355	2036	1941	343161	125412
	Geo Mean			9	8	9	10	2582	1945	1277	1125	104205	86528
pool			4										

78

Example 29

C57Bl/6 mice were immunized transcutaneously with
CT (azide-free, Calbiochem) as described above on the
shaved back of the mouse. Mice were challenged using

5   a lethal challenge model 6 weeks after immunization
(Mallet et al, Immunoprophylactic efficacy of nontoxic
mutants of *Vibrio cholerae* toxin (CTK63) and
Escherichia coli heat-labile toxin (LTK63) in a mouse
cholera toxin intranasal challenge model, submitted to

10 *Immunology Letters*). In the challenge, mice were
given 20 μg of CT (Calbiochem, azide free) dissolved
in saline intranasally via a plastic pipette tip while
anesthetized with 20 μl of ketamine-rompin. In trial
#1, 12/15 immunized mice survived the challenge after

15 14 days and 1/9 unimmunized control mice survived.
Five control mice were lost prior to challenge due to
anesthesia. Mice in challenge #1 had anti-CT serum
antibodies of 15,000 ELISA units (geometric mean), and
five immunized mice sacrificed at the time of

20 challenge had lung wash IgG detected in 5/5 mice.
Lung washes were collected as described above.

The immunization and challenge was repeated with
naïve C57Bl/6 mice and 7/16 immunized mice survived
the challenge, while only 2/17 unimmunized mice

25 survived the challenge. Immunized mice in challenge
#2 had anti-CT IgG antibodies of 41,947 ELISA units
(geometric mean). Lung washes from five mice
sacrificed at the time of challenge demonstrated both
anti-CT IgG and IgA (Table 24). Stool samples from

30 8/9 mice demonstrated both anti-CT IgG and IgA (Table
25). Stool samples were collected fresh from animals
spontaneously defecating at the time of challenge.
The stools were frozen at -20°C. At the time of ELISA,
the stools were thawed, homogenized in 100 μl of PBS,

35 centrifuged and ELISA run on the supernatant. The
combined survival rate among immunized mice was 19/31

or 61% whereas the combined survival rate among unimmunized mice was 3/26 or 12%.

Table 24. Lung wash anti-Cholera toxin IgG and IgA antibody titers

Sample Dilution	Animal Identification Number				
	8969	8970	8971	8972	8995
	IgG (H+L) anti-CT (Optical Density)				
1:10	3.613	3.368	3.477	3.443	3.350
1:20	3.302	3.132	3.190	3.164	3.166
1:40	3.090	2.772	2.825	2.899	2.692
1:80	2.786	2.287	2.303	2.264	2.086
1:160	2.041	1.570	1.613	1.624	1.441
1:320	1.325	0.971	1.037	1.041	0.965
1:640	0.703	0.638	0.601	0.644	0.583
1:1280	0.434	0.382	0.350	0.365	0.364
	IgA anti-CT (Optical Density)				
1:2	1.235	2.071	2.005	2.115	1.984
1:4	1.994	1.791	1.836	1.85	1.801
1:8	1.919	1.681	2.349	1.796	1.742
1:16	1.8	1.457	1.577	1.614	1.536
1:32	1.503	1.217	1.36	1.523	1.23
1:64	1.189	0.863	1.044	1.101	0.88
1:128	0.814	0.57	0.726	0.74	0.595
1:356	0.48	0.334	0.436	0.501	0.365

80

Table 25. Stool anti-Cholera toxin IgG and IgA antibody titers

Sample Dilution	mouse identification number (immunization group)											
	8985 (CT)	8997 (CT)	8987 (CT)	8990 (CT)	8977 (CT)	8976 (CT)	8975 (CT)	8988 (CT)	8994 (none)	8979 (none)	9000 (none)	8983 (none)
IgG (H+L) anti-CT (optical density)												
1:10	1.01	1.91	2.33	0.03	0.74	1.98	1.20	1.45	0.09	0.05	0.02	0.18
1:20	0.42	0.94	1.26	-	0.31	1.19	0.50	0.91	0.04	-	-	0.08
1:40	0.20	0.46	0.68	-	0.12	0.58	0.24	0.49	-	-	-	0.02
1:80	0.10	0.21	0.34	-	0.05	0.31	0.09	0.25	-	-	-	-
1:160	0.03	0.09	0.18	-	0.02	0.14	0.05	0.12	-	-	-	-
IgA Anti-CT (optical density)												
1:4	0.32	1.14	0.43	0.00	0.19	1.00	0.58	1.21	0.02	-	0.07	-
1:8	0.16	0.67	0.24	-	0.08	0.56	0.36	0.77	-	-	-	-
1:16	0.08	0.33	0.11	-	0.03	0.27	0.17	0.40	-	-	-	-
1:32	0.06	0.16	0.05	-	0.03	0.12	0.08	0.20	-	-	-	-
1:64	0.01	0.07	0.03	-	-	0.05	0.03	0.10	-	-	-	-

81

Example 30

C57Bl/6 female mice were obtained from Charles
River Laboratories. The mice were immunized with 200
µg ovalbumin (OVA) (Sigma, lot #14H7035, stock
5   concentration of 2 mg/ml in PBS) and 50 µg Cholera
Toxin (List Biologicals, lot #101481B, stock
concentration of 5 mg/ml). A Packard Cobra Gamma
Counter was used (serial #102389) to measure the
amount of ^{51}Cr released.
10   C57Bl/6 mice were anesthetized with 0.03 ml of
ketamine-rompin and shaved on the dorsum with a
clipper, without traumatizing the skin, and were
rested for 24 hours. The mice were anesthetized then
immunized at 0 and 28 days with 150 µl of immunizing
15   solution placed on the shaved skin over a 2 cm^2 area
for 2 hours. The mice were then wiped twice with wet
gauze. The mice exhibited no adverse effects from
either anesthesia, immunization, or the washing
procedure. This procedure was repeated weekly for
20   three weeks.

Splenic lymphocytes were collected one week after
boosting immunization. Cells were cultured in vitro
in RPMI-1640 and 10% FBS (with penicillin-
streptomycin, glutamine, non-essential amino acids,
25   sodium pyruvate and 2-mercaptoethanol) for 6 days with
the addition of 5% rat concanavalin A supernatant as a
source of IL-2, with or without antigen. Target cells
consist of syngeneic (H-2^b) EL4 cells alone and EL4
cells pulsed with the CTL peptide SINFEKKL, allogeneic
30   (H-2k) L929 cells and EG7 cells. The target cells (1
x 10^6 cells per well) were labeled for 1 h with 0.1 mCi
per well ^{51}Cr (Na$_2$CrO$_4$ source, Amersham) and were added
to effector cells at ratios ranging from 3:1 to 100:1.
The cell mixtures were incubated in 96-well round
35   bottom tissue culture plates (Costar, catalog #3524)
in 0.2 ml complete RPMI-1640, 10% FBS medium for 5 h

at 37°C in a 5% CO_2 humidified atmosphere. At the end
of the 5 h culture, the supernatants were absorbed by
cotton wicks and processed for the determination of
51Cr release. Specific lysis was determined as:

5 % Specific Lysis = 100 x [(experimental release –
spontaneous release) / (maximal release – spontaneous
release)].

As shown in Table 26, part 1 CTLs were detected
against the EL4 peptide pulsed cells at an E:T ratio
10 of 100:1 for the group immunized with CT+OVA. CTL
assays are not positive if the percent specific lysis
is not above 10% or clearly above the media-stimulated
effectors background percentage lysis. Similarly, as
shown in Table 26, part 2 CTLs were detected against
15 the EG7 (OVA transfected cells) at an E:T ratio of
100:1 for the group immunized with CT+OVA. Thus, CT
adjuvanted for the production of CTLs via the
transcutaneous route.

20
Table 26. OVA-specific CTL induced Transcutaneously

Part 1 - Target Cells: EL4+Peptide

	CT+OVA	CT+OVA	Imm. Group CT	CT	OVA	OVA
			Stimulated with			
E:T Ratio	Media	Ova	Media	Ova	Media	Ova
100:1	9.5	13.1	11.1	12.5	23.1	21.5
30:1	6.9	6.8	5.9	8.9	14.2	10.7
10:1	4.9	3.5	3.5	8.5	7.7	5.2

Part 2 - Target Cells: EG7 (OVA Transfected)
35

	CT+OVA	CT+OVA	Imm. Group CT	CT	OVA	OVA
			Stimulated with			
E:T Ratio	Media	Ova	Media	Ova	Media	Ova
100:1	10.6	17.6	14.5	16.8	23.8	26
30:1	4.9	9.5	8.2	10.1	13.6	10.7
10:1	6.4	4.4	4	5	7.3	4.2

45

Example 31

C57B1/6 mice (n=6) were immunized
transcutaneously as described above for "Immunization
Procedure". Mice were immunized at 0 and 4 weeks with
100 µl saline containing 100 µg Cholera toxin (List
5   Biologicals, catalog #101B, lot #10149CB) and 250 µg
of ovalbumin protein (Sigma, albumin chicken egg,
Grade V catalog #A5503, lot #14H7035).
       Single cell suspensions were prepared from
spleens harvested from animals at eight weeks after
10   the first immunization. Splenocytes were set up in
culture at 8×10^5 cells per well in a 200 µl volume
containing OVA protein or the irrelevant protein
Conalbumin at the concentrations indicated. Cultures
were incubated for 72 hours at 37°C in a CO_2 incubator
15   at which time 0.5 µCi/well of ^3H thymidine was added to
each well. Twelve hours later, proliferation was
assessed by harvesting the plates and quantitating
incorporated radiolabelled thymidine by liquid
scintillation counting. Raw values of ^3H incorporation
20   are indicated in cpm and the fold increase (cpm
experimental / cpm media) is indicated to the left of
each sample. Fold increases greater than three are
considered significant.
       Significant proliferation was only detected when
25   the splenocytes were stimulated with the protein,
Ovalbumin, to which the animals had been immunized
with in vivo and not with the irrelevant protein
conalbumin. Thus transcutaneous immunization with
Cholera toxin and ovalbumin protein induces antigen
30   specific proliferation of splenocytes in vitro
indicating that a cellular immune response is evoked
by this method.

Table 27. Antigen specific proliferation of spleen cells from animals immunized with Cholera toxin (CT) and Ovalbumin (OVA)

Immunization group	Concentration of *in vitro* stimuli	media		OVA protein	
Conalbumin		3-H incorporation cpm	fold increase	3-H incorporation cpm	fold increase
CT/OVA	10 µg/ml1427	13450	9.4	3353	2.3
	1 µg/ml	4161	2.9	2638	1.8
	0.1 µg/ml	2198	1.5	2394	1.7
	0.01 µg/ml	3419	2.4	2572	1.8

The disclosures of all patents, as well as all other printed documents, cited in this specification are incorporated herein by reference in their entirety. Such references are cited as indicative of the skill in the art.

While the present invention has been described in connection with what is presently considered to be practical and preferred embodiments, it is understood that the present invention is not to be limited or restricted to the disclosed embodiments but, on the contrary, is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

Thus, it is to be understood that variations in the described invention will be obvious to those skilled in the art without departing from the novel aspects of the present invention and such variations are intended to come within the scope of the claims below.

REFERENCES

Aiba and Katz (1990) Immunology 145:2791-2796

Alving et al (1986) Vaccine 4:166-172.

Alving and Wassef (1994) AIDS Res Hum Retro 10 (suppl. 2):S91-S94.

Alving et al (1993) In: *Liposome Technology*, vol. 3, pp. 317-343.

85

Antel et al (1996) Nature Medicine 2:1074-1075.

Ausubel et al (1996) *Current Protocols in Molecular Biology*.

Bacci et al (1997) Eur J Immunol 27:442-448.

5 Bathurst et al (1993) Vaccine 11:449-456.

Bellinghausen et al (1996) J Invest Dermatol 107:582-588.

Blauvelt et al (1995) J Invest Dermatol 104:293-296.

Blum (1995) Digestion 56:85-95.

10 Bodanszky (1993) *Peptide Chemistry*.

Bos (1997) Clin Exp Immunol 107 (suppl. 1):3-5.

Bowen et al (1994) Immunology 81:338-342.

Burnette et al (1994) In: *Bioprocess Technology*, pp. 185-203.

15 Caughman et al (1986) Proc Natl Acad Sci USA 83:7438-7442.

Chang et al (1989) Proc Natl Acad Sci USA 86:6343-6347.

Chang et al (1992) J Immunol 139:548-555.

20 Chang et al (1994) J Immunol 152:3483-3490.

Craig and Cuatrecasas (1975) Proc Natl Acad Sci USA 72:3384-2288.

Dahl (1996) In: *Clinical Immunodermatology*, 3rd Ed., pp. 345-352.

25 Delenda et al (1994) J Gen Virol 75:1569-1578.

Deprez et al (1996) Vaccine 14:375-382.

Deutscher (1990) *Guide to Protein Purification*.

Dragunsky et al (1992) Vaccine 10:735-736.

Elson and Dertzbaugh (1994) In: *Handbook of Mucosal*

30 *Immunology*, p. 391.

Enk et al (1993) J Immunol 150:3698-3704.

Fonseca et al (1994) Vaccine 12:279-285.

Frankenburg et al (1996) Vaccine 14:923-929.

Fries et al (1992a) Proc Natl Acad Sci USA 89:358-362.

35 Fries et al (1992b) Infect Immunity 60:1834-1839.

Glenn et al (1995) Immunol Lett 47:73-78.

Goeddel (1990) *Gene Expression Technology*.

86

Gregoriadis (1993) *Liposome Preparation and Related Techniques*, 2nd Ed.

Herrington et al (1991) Am J Trop Med Hyg 45:695-701.

Jahrling et al (1996) Arch Virol Suppl 11:135-140.

Janeway and Travers (1996) *Immunobiology*.

Janson and Ryden (1989) *Protein Purification*.

Katkov (1996) Med Clin North Am 80:189-200.

Khusmith et al (1991) Science 252:715-718.

Kounnas et al (1992) J Biol Chem 267:12420-12423.

Kriegler (1990) *Gene Transfer and Expression*.

Kripke et al (1990) J Immunol 145:2833-2838.

Krueger and Barbieri (1995) Clin Microbiol Rev 8:34-47.

Lee and Chen (1994) Infect Immunity 62:3594-3597.

Leung (1997) Clin Exp Immunol 107 (Suppl. 1):25-30.

Lycke and Holmgren (1986) Immunology 59:301-308.

Lieberman and Greenberg (1996) Adv Pediatr Infect Dis 11:333-363.

Malik et al (1991) Proc Natl Acad Sci USA 88:3300-3304.

Mast and Krawczynski (1996) Annu Rev Med 47:257-266.

Migliorini et al (1993) Eur J Immunol 23:582-585.

Morein and Simons (1985) Vaccine 3:83-93.

Murray (1991) *Gene Transfer and Expression Protocols*.

Nohria and Rubin (1994) Biotherapy 7:261-269.

Paul and Cevc (1995) Vaccine Res 3:145-164.

Paul et al (1995) Eur J Immunol 25:3521-3524, 1995.

Paul and Seder (1994) Cell 76:241-251.

Peguet-Navarro et al (1995) Adv Exp Med Biol 378:359-361.

Pessi et al (1991) Eur J Immunol 24:2273-2276.

Pierce (1978) J Exp Med 148:195-206.

Pierce and Reynolds (1974) J Immunol 113:1017-1023.

Plotkin and Mortimer (1994) *Vaccines*.

Porgador et al (1997) J Immunol 158:834-841.

Rappuoli et al (1995) Int Archiv Allergy Immunol 108:327-333.

87

Rappuoli et al (1996) Adv Exp Med Biol 397:55-60.

Ribi et al (1988) Science 239:1272-1276.

Richards et al (1995) In: *Vaccine Design*.

Roberts and Walker (1993) In: *Pharmaceutical Skin*

5 *Penetration Enhancement*.

Saloga et al (1996) Exp Dermatol 5:65-71.

Schneerson et al (1996) Lancet 348:1289-1292.

Schuler and Steinman (1985) J Exp Med 161:526-546.

Schwarzenberger and Udey (1996) J Invest Dermatol

10 106:553-558.

Scopes (1993) *Protein Purification*.

Seder and Paul (1994) Annu Rev Immunol 12:635-673.

Shafara et al (1995) Ann Intern Med 125:658-668.

Shankar et al (1996) Cell Immunol 171:240-245.

15 Skeiky et al (1995) J Exp Med 181:1527-1537.

Smedile et al (1994) Prog Liver Dis 12:157-175.

Smucny et al (1995) Am J Trop Med Hyg 53:432-437.

Stacey et al (1996) J Immunol 157:2116-2122.

Stingl et al (1989) Immunol Ser 46:3-42.

20 Streilein and Grammer (1989) J Immunol 143:3925-3933.

Summers and Smith (1987) *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedure*.

Tam (1988) Proc Natl Acad Sci USA 85:5409-5413.

Tew et al (1997) Immunol Rev 156:39-52.

25 Trach et al (1997) Lancet 349:231-235.

Udey (1997) Clin Exp Immunol 107 (Suppl. 1):6-8.

Vandenbark et al (1996) Nature Medicine 2:1109-1115.

Vreden et al (1991) Am J Trop Med Hyg 45:533-538.

Wang et al (1995) J Immunol 154:2784-2793.

30 Wertz (1992) In: *Liposome Dramatics*, pp. 38-43.

White et al (1993) Vaccine 11:1341-1346.

Wiesmueller et al (1991) Immunology 72:109-113.

Wisdom (1994) *Peptide Antigens*.

Zhang et al (1995) Infect Immun 63:1349-1355.

88

WHAT WE CLAIM IS:

1.   A formulation for transcutaneous
immunization comprising an antigen and an adjuvant,
wherein application of the formulation to intact skin
induces an immune response specific for the antigen
without perforating the skin.

2.   The formulation of claim 1, which further
comprises a dressing to form a patch for
transcutaneous immunization.

3.   The formulation of claim 2, wherein the
dressing is an occlusive dressing.

4.   The formulation of claim 2, wherein the
dressing covers more than one draining lymph node
field.

5.   The formulation of claim 1, wherein the
adjuvant enhances presentation of the antigen to a
lymphocyte.

6.   The formulation of claim 1, wherein the
adjuvant activates an antigen presenting cell.

7.   The formulation of claim 6, wherein the
antigen presenting cell is a Langerhans cell or a
dermal dendritic cell.

8.   The formulation of claim 1, wherein the
adjuvant increases major histocompatibility complex
class II expression on an antigen presenting cell.

9.   The formulation of claim 8, wherein the
antigen presenting cell is a Langerhans cell or a
dermal dendritic cell.

89

10.   The formulation of claim 1, wherein the adjuvant causes an antigen presenting cell underlying an application site to migrate to a draining lymph
5   node.

11.   The formulation of claim 10, wherein the antigen presenting cell is a Langerhans cell or a dermal dendritic cell.
10

12.   The formulation of claim 1, wherein the adjuvant signals a Langerhans cell to mature into a dendritic cell.

15   13.   The formulation of claim 1, which consists essentially of antigen and adjuvant.

14.   The formulation of claim 1, wherein a component of the formulation is both antigen and
20   adjuvant.

15.   The formulation of claim 1, which is an aqueous solution.

25.   16.   The formulation of claim 1, which does not include an organic solvent.

17.   The formulation of claim 1, which does not include a penetration enhancer.
30

18.   The formulation of claim 1, which is formed as an emulsion.

19.   The formulation of claim 1, wherein the
35   antigen is derived from a pathogen selected from the group consisting of bacterium, virus, fungus, and parasite.

20. The formulation of claim 1, wherein the antigen is a tumor antigen.

5   21. The formulation of claim 1, wherein the antigen is an autoantigen.

22. The formulation of claim 1, wherein the antigen is an allergan.

10

23. The formulation of claim 1, wherein the antigen is greater than 500 daltons molecular weight.

24. The formulation of claim 1, which includes

15   at least two different separate antigens.

25. The formulation of claim 1, wherein the antigen is provided as a nucleic acid encoding the antigen.

20

26. The formulation of claim 25, wherein the nucleic acid is non-integrating and non-replicating.

27. The formulation of claim 25, wherein the

25   nucleic acid further comprises a regulatory region operably linked to the sequence encoding the antigen.

28. The formulation of claim 25, which does not include a penetration enhancer, viral particle,

30   liposome, or charged lipid.

29. The formulation of any one of claims 1-28, wherein the adjuvant is an ADP-ribosylating exotoxin.

35   30. The formulation of claim 29, wherein the ADP-ribosylating exotoxin is cholera toxin or a functional derivative thereof.

31. The formulation of claim 29, wherein the ADP-ribosylating exotoxin is *E. coli* heat-labile enterotoxin, pertussis toxin, or a functional

5   derivative thereof.

32. The formulation of claim 29, wherein the adjuvant is provided as a nucleic acid encoding an ADP-ribosylating exotoxin.

10

33. The formulation of any one of claims 1-28, wherein application of the formulation does not involve physical, electrical, or sonic energy which perforates the intact skin.

15

34. The formulation of any one of claims 1-28, wherein the immune response is not an allergic reaction, dermatitis, or an atopic reaction.

20   35. Making the formulation of any of the foregoing claims.

36. Using the formulation of any of the foregoing claims to induce an immune response in a
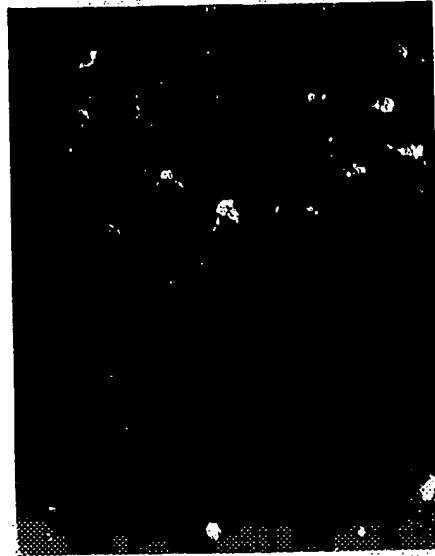
25   non-human animal.

FIG. 1A

FIG. 1B

FIG. 1C

FIG. 1D

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :A01N 37/18; A61F 13/00; A61K 39/00, 9/127, 9/52, 9/56

US CL   : 424/184.1, 422, 450, 457, 459; 514/2

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   424/184.1, 422, 450, 457, 459; 514/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG EMBASE WPID LIFESCI BIOSIS MEDLINE CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PAUL et al. Noninvasive Administration of Protein Antigens: Transdermal Immunization with Bovine Serum Albumin in Transfersomes. Vaccine Research. 01 March 1995, Vol. 4, Number 3, pages 145-164, see entire document.	1-36
Y	PAUL et al. Transdermal immunization with large proteins by means of ultradeformable drug carriers. European J. Immunology. 01 August 1995, Vol. 25, No. 8, pages 3521-3524, see entire document.	1-36

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
03 JANUARY 1998	10 FEB 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer PATRICK NOLAN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)☆